abnormalities including but not limited to translocations, transversions, monosomies, trisomy 21, trisomy 18, trisomy 13, other anueoplodies, deletions, additions, amplifications, translocations and rearrangements.

EXAMPLE 6

Genomic DNA was obtained from four individuals after informed consent was obtained. Six SNPs on chromosome 13 (TSC0837969, TSC0034767, TSC1130902, TSC0597888, TSC0195492, TSC0607185) were analyzed using the template DNA. Information regarding these SNPs can be found at the following website www.snp.chsl.org/snpsearch.shtml; website active as of February 11, 2003).

A single nucleotide labeled with one fluorescent dye was used to genotype the individuals at the six selected SNP sites. The primers were designed to allow the six SNPs to be analyzed in a single reaction.

Preparation of Template DNA

The template DNA was prepared from a 9 ml sample of blood obtained by venipuncture from a human volunteer with informed consent. Template DNA was isolated using the QIAmp DNA Blood Midi Kit supplied by QIAGEN (Catalog number 51183). The template DNA was isolated as per instructions included in the kit.

Design of Primers

SNP TSC0837969 was amplified using the following primer set:

First primer:

5' GGGCTAGTCTCCGAATTCCACCTATCCTACCAAATGTC 3'

Second primer:

5' TAGCTGTAGTTAGGGACTGTTCTGAGCAC 3'

The first primer had a biotin tag at the 5' end and contained a restriction enzyme recognition site for EcoRI. The first primer was designed to anneal 44 bases from the locus of interest. The second primer contained a restriction enzyme recognition site for BsmF I.

SNP TSC0034767 (50) was amplified using the following primer set:

First primer:

5' CGAATGCAAGGCGAATTCGTTAGTAATAACACAGTGCA 3'

Second primer:

5' AAGACTGGATCCGGGACCATGTAGAATAC 3'

The first primer had a biotin tag at the 5' end and contained a restriction enzyme recognition site for EcoRI. The first primer was designed to anneal 50 bases from the locus of interest. The second primer contained a restriction enzyme recognition site for BsmF I.

SNP TSC1130902 (60) was amplified using the following primer set:

First primer:

5' TCTAACCATTGCGAATTCAGGGCAAGGGGGGTGAGATC 3'

Second primer:

5' TGACTTGGATCCGGGACAACGACTCATCC 3'

The first primer had a biotin tag at the 5' end and contained a restriction enzyme recognition site for EcoRI. The first primer was designed to anneal 60 bases from the

locus of interest. The second primer contained a restriction enzyme recognition site for BsmF I.

SNP TSC0597888 (70) was amplified using the following primer set:

First primer:

5' ACCCAGGCGCCAGAATTCTTTAGATAAAGCTGAAGGGA 3'

Second primer:

5' GTTACGGGATCCGGGACTCCATATTGATC 3'

The first primer had a biotin tag at the 5' end and contained a restriction enzyme recognition site for EcoRI. The first primer was designed to anneal 70 bases from the locus of interest. The second primer contained a restriction enzyme recognition site for BsmF I.

SNP TSC0195492 (80) was amplified using the following primer set:

First primer:

5'CGTTGGCTTGAGGAATTCGACCAAAAGAGCCAAGAGAA

Second primer:

5' AAAAAGGGATCCGGGACCTTGACTAGGAC 3'

The first primer had a biotin tag at the 5' end and contained a restriction enzyme recognition site for EcoRI. The first primer was designed to anneal 80 bases from the

locus of interest. The second primer contained a restriction enzyme recognition site for BsmF I.

SNP TSC0607185 (90) was amplified using the following primer set:

First primer:

5' ACTTGATTCCGTGAATTCGTTATCAATAAATCTTACAT 3'

Second primer:

5' CAAGTTGGATCCGGGACCCAGGGCTAACC 3'

The first primer had a biotin tag at the 5' end and contained a restriction enzyme recognition site for EcoRI. The first primer was designed to anneal 90 bases from the locus of interest. The second primer contained a restriction enzyme recognition site for BsmF I.

All loci of interest were amplified from the template genomic DNA using the polymerase chain reaction (PCR, U.S. Patent Nos. 4,683,195 and 4,683,202, incorporated herein by reference). In this example, the loci of interest were amplified in separate reaction tubes but they could also be amplified together in a single PCR reaction. For increased specificity, a "hot-start" PCR was used. PCR reactions were performed using the HotStarTaq Master Mix Kit supplied by QIAGEN (catalog number 203443). The amount of template DNA and primer per reaction can be optimized for each locus of interest but in this example, 40 ng of template human genomic DNA and 5 μM of each primer were used. Forty cycles of PCR were performed. The following PCR conditions were used:

(1) 95°C for 15 minutes and 15 seconds;
(2) 37°C for 30 seconds;
(3) 95°C for 30 seconds;
(4) 57°C for 30 seconds;
(5) 95°C for 30 seconds;
(6) 64°C for 30 seconds;

(7) 95°C for 30 seconds;

(8) Repeat steps 6 and 7 thirty nine (39) times;

(9) 72°C for 5 minutes.

In the first cycle of PCR, the annealing temperature was about the melting temperature of the 3′ annealing region of the second primers, which was 37°C. The annealing temperature in the second cycle of PCR was about the melting temperature of the 3' region, which anneals to the template DNA, of the first primer, which was 57°C. The annealing temperature in the third cycle of PCR was about the melting temperature of the entire sequence of the second primer, which was 64°C. The annealing temperature for the remaining cycles was 64°C. Escalating the annealing temperature from TM1 to TM2 to TM3 in the first three cycles of PCR greatly improves specificity. These annealing temperatures are representative, and the skilled artisan will understand the annealing temperatures for each cycle are dependent on the specific primers used.

The temperatures and times for denaturing, annealing, and extension, can be optimized by trying various settings and using the parameters that yield the best results. In this example, the first primer was designed to anneal at various distances from the locus of interest. The skilled artisan understands that the annealing location of the first primer can be 5-10, 11-15, 16-20, 21-25, 26-30, 31-35, 36-40, 41-45, 46-50, 51-55, 56-60, 61-65, 66-70, 71-75, 76-80, 81-85, 86-90, 91-95, 96-100, 101-105, 106-110, 111-115, 116-120, 121-125, 126-130, 131-140, 1410-160, 1610-180, 1810-200, 2010-220, 2210-240, 2410-260,. 2610-280,. 2810-300, 3010-350, 3510-400, 4010-450, 450-500, or greater than 500 bases from the locus of interest.

Purification of Fragment of Interest

The PCR products were separated from the genomic template DNA. After the PCR reaction, 1//4 of the volume of each PCR reaction from one individual was mixed together in a well of a Streptawell, transparent, High-Bind plate from Roche Diagnostics GmbH (catalog number 1 645 692, as listed in Roche Molecular Biochemicals, 2001 Biochemicals Catalog). The first primers contained a 5' biotin tag so the PCR products bound to the Streptavidin coated wells while the genomic template DNA did not. The streptavidin binding reaction was performed using a Thermomixer (Eppendorf) at 1000 rpm for 20 min. at 37°C. Each well was aspirated to remove unbound material, and

washed three times with 1X PBS, with gentle mixing (Kandpal et al., Nucl. Acids Res. 18:1789-1795 (1990); Kaneoka et al., Biotechniques 10:30-34 (1991); Green et al., Nucl. Acids Res. 18:6163-6164 (1990)).

Restriction Enzyme Digestion of Isolated Fragments

The purified PCR products were digested with the restriction enzyme BsmF I, which binds to the recognition site incorporated into the PCR products from the second primer. The digests were performed in the Streptawells following the instructions supplied with the restriction enzyme. After digestion, the wells were washed three times with PBS to remove the cleaved fragments.

Incorporation of Labeled Nucleotide

The restriction enzyme digest with BsmF I yielded a DNA fragment with a 5' overhang, which contained the SNP site or locus of interest and a 3' recessed end. The 5' overhang functioned as a template allowing incorporation of a nucleotide or nucleotides in the presence of a DNA polymerase.

Below, a schematic of the 5' overhang for SNP TSC0837969 is shown. The entire DNA sequence is not reproduced, only the portion to demonstrate the overhang (where R indicates the variable site).

	5' TTAA				
	3' AATT	R	A	C	A
	Overhang position	1	2	3	4

The observed nucleotides for TSC0837969 on the 5' sense strand (here depicted as the top strand) are adenine and guanine. The third position in the overhang on the antisense strand corresponds to cytosine, which is complementary to guanine. As this variable site can be adenine or guanine, fluorescently labeled ddGTP in the presence of unlabeled dCTP, dTTP, and dATP was used to determine the sequence of both alleles. The fill-in reactions for an individual homozygous for guanine, homozygous for adenine or heterozygous are diagrammed below.

Homozygous for guanine at TSC 0837969:

Allele 1	5' TTAA	G*			
	3' AATT	C	A	C	A
Overhang position		1	2	3	4

Allele 2	5' TTAA	G*			
	3' AATT	C	A	C	A
Overhang position		1	2	3	4

Labeled ddGTP is incorporated into the first position of the overhang. Only one signal is seen, which corresponds to the molecules filled in with labeled ddGTP at the first position of the overhang.

Homozygous for adenine at TSC 0837969:

Allele 1	5' TTAA	A	T	G*	
	3' AATT	T	A	C	A
Overhang position		1	2	3	4

Allele 2	5' TTAA	A	T	G*	
	3' AATT	T	A	C	A
Overhang position		1	2	3	4

Unlabeled dATP is incorporated at position one of the overhang, and unlabeled dTTP is incorporated at position two of the overhang. Labeled ddGTP was incorporated at position three of the overhang. Only one signal will be seen; the molecules filled in with ddGTP at position 3 will have a different molecular weight from molecules filled in at position one, which allows easy identification of individuals homozygous for adenine or guanine.

Heterozygous at TSC0837969:

Allele 1	5' TTAA	G*

	3' AATT	C	A	C	A
Overhang position		1	2	3	4
Allele 2	5' TTAA	A	T	G*	
	3' AATT	T	A	C	A
Overhang position		1	2	3	4

Two signals will be seen; one signal corresponds to the DNA molecules filled in with ddGTP at position 1, and a second signal corresponding to molecules filled in at position 3 of the overhang. The two signals can be separated using any technique that separates based on molecular weight including but not limited to gel electrophoresis.

Below, a schematic of the 5' overhang for SNP TSC0034767 is shown. The entire DNA sequence is not reproduced, only the portion to demonstrate the overhang (where R indicates the variable site).

A	C	A	R	GTGT 3'
				CACA 5'
4	3	2	1	Overhang Position

The observed nucleotides for TSC0034767 on the 5' sense strand (here depicted as the top strand) are cytosine and guanine. The second position in the overhang corresponds to adenine, which is complementary to thymidine. The third position in the overhang corresponds to cytosine, which is complementary to guanine. Fluorescently labeled ddGTP in the presence of unlabeled dCTP, dTTP, and dATP is used to determine the sequence of both alleles.

In this case, the second primer anneals from the locus of interest, and thus the fill-in reaction occurs on the anti-sense strand (here depicted as the bottom strand). Either the sense strand or the antisense strand can be filled in depending on whether the second primer, which contains the type IIS restriction enzyme recognition site, anneals upstream or downstream of the locus of interest.

Below, a schematic of the 5' overhang for SNP TSC1130902 is shown. The entire DNA sequence is not reproduced, only a portion to demonstrate the overhang (where R indicates the variable site).

	5' TTCAT				
	3' AAGTA	R	T	C	C
Overhang position		1	2	3	4

The observed nucleotides for TSC1130902 on the 5' sense strand (here depicted as the top strand) are adenine and guanine. The second position in the overhang corresponds to a thymidine, and the third position in the overhang corresponds to cytosine, which is complementary to guanine.

Fluorescently labeled ddGTP in the presence of unlabeled dCTP, dTTP, and dATP is used to determine the sequence of both alleles.

Below, a schematic of the 5' overhang for SNP TSC0597888 is shown. The entire DNA sequence is not reproduced, only the portion to demonstrate the overhang (where R indicates the variable site).

T	C	T	R	ATTC 3'
				TAAG 5'
4	3	2	1	Overhang position

The observed nucleotides for TSC0597888 on the 5' sense strand (here depicted as the top strand) are cytosine and guanine. The third position in the overhang corresponds to cytosine, which is complementary to guanine. Fluorescently labeled ddGTP in the presence of unlabeled dCTP, dTTP, and dATP is used to determine the sequence of both alleles.

Below, a schematic of the 5' overhang for SNP TSC0607185 is shown. The entire DNA sequence is not reproduced, only the portion to demonstrate the overhang (where R indicates the variable site).

C	C	T	R	TGTC 3'
				ACAG 5'
4	3	2	1	Overhang position

The observed nucleotides for TSC0607185 on the 5' sense strand (here depicted as the top strand) are cytosine and thymidine. In this case, the second primer anneals from the locus of interest, which allows the anti-sense strand to be filled in. The anti-sense strand (here depicted as the bottom strand) will be filled in with guanine or adenine.

The second position in the 5' overhang is thymidine, which is complementary to adenine, and the third position in the overhang corresponds to cytosine, which is complementary to guanine. Fluorescently labeled ddGTP in the presence of unlabeled dCTP, dTTP, and dATP is used to determine the sequence of both alleles.

Below, a schematic of the 5' overhang for SNP TSC0195492 is shown. The entire DNA sequence is not reproduced, only the portion to demonstrate the overhang.

	5' ATCT				
	3' TAGA	R	A	C	A
Overhang position		1	2	3	4

The observed nucleotides at this site are cytosine and guanine (here depicted as the top strand) . The second position in the 5' overhang is adenine, which is complementary to thymidine, and the third position in the overhang corresponds to cytosine, which is complementary to guanine. Fluorescently labeled ddGTP in the presence of unlabeled dCTP, dTTP, and dATP is used to determine the sequence of both alleles.

As demonstrated above, the sequence of both alleles of the six SNPs can be determined by labeling with ddGTP in the presence of unlabeled dATP, dTTP, and dCTP. The following components were added to each fill in reaction: 1 µl of fluorescently labeled ddGTP, 0.5 µl of unlabeled ddNTPs (40 µM), which contained all nucleotides except guanine, 2 µl of 10X sequenase buffer, 0.25 µl of Sequenase, and water as needed for a 20µl reaction. The fill in reaction was performed at 40°C for 10 min. Non-fluorescently labeled ddNTP was purchased from Fermentas Inc. (Hanover, MD). All other labeling reagents were obtained from Amersham (Thermo Sequenase Dye Terminator Cycle Sequencing Core Kit, US 79565).

After labeling, each Streptawell was rinsed with 1X PBS (100 µl) three times. The "filled in" DNA fragments were then released from the Streptawells by digestion with the restriction enzyme EcoRI, according to the manufacturer's instructions that were

supplied with the enzyme. Digestion was performed for 1 hour at 37 °C with shaking at 120 rpm.

Detection of the Locus of Interest

After release from the streptavidin matrix, the sample was loaded into a lane of a 36 cm 5% acrylamide (urea) gel (BioWhittaker Molecular Applications, Long Ranger Run Gel Packs, catalog number 50691). The sample was electrophoresed into the gel at 3000 volts for 3 min. The gel was run for 3 hours on a sequencing apparatus (Hoefer SQ3 Sequencer). The gel was removed from the apparatus and scanned on the Typhoon 9400 Variable Mode Imager. The incorporated labeled nucleotide was detected by fluorescence.

As shown in FIG. 11, the template DNA in lanes 1 and 2 for SNP TSC0837969 is homozygous for adenine. The following fill-in reaction was expected to occur if the individual was homozygous for adenine:

Homozygous for adenine at TSC 0837969:

5' TTAA	A	T	G*	
3' AATT	T	A	C	A
Overhang position	1	2	3	4

Unlabeled dATP was incorporated in the first position complementary to the overhang. Unlabeled dTTP was incorporated in the second position complementary to the overhang. Labeled ddGTP was incorporated in the third position complementary to the overhang. Only one band was seen, which migrated at about position 46 of the acrylamide gel. This indicated that adenine was the nucleotide filled in at position one. If the nucleotide guanine had been filled in, a band would be expected at position 44.

However, the template DNA in lanes 3 and 4 for SNP TSC0837969 was heterozygous. The following fill-in reactions were expected if the individual was heterozygous:

Heterozygous at TSC0837969:

Allele 1 5' TTAA G*

	3' AATT	C	A	C	A
	Overhang position	1	2	3	4
	Allele 25' TTAA	A	T	G*	
	3' AATT	T	A	C	A
	Overhang position	1	2	3	4

Two distinct bands were seen; one band corresponds to the molecules filled in with ddGTP at position 1 complementary to the overhang (the G allele), and the second band corresponds to molecules filled in with ddGTP at position 3 complementary to the overhang (the A allele). The two bands were separated based on the differences in molecular weight using gel electrophoresis. One fluorescently labeled nucleotide ddGTP was used to determine that an individual was heterozygous at a SNP site. This is the first use of a single nucleotide to effectively detect the presence of two different alleles.

For SNP TSC0034767, the template DNA in lanes 1 and 3 is heterozygous for cytosine and guanine, as evidenced by the two distinct bands. The lower band corresponded to ddGTP filled in at position 1 complementary to the overhang. The second band of slightly higher molecular weight corresponded to ddGTP filled in at position 3, indicating that the first position in the overhang was filled in with unlabeled dCTP, which allowed the polymerase to continue to incorporate nucleotides until it incorporated ddGTP at position 3 complementary to the overhang. The template DNA in lanes 2 and 4 was homozygous for guanine, as evidenced by a single band of higher molecular weight than if ddGTP had been filled in at the first position complementary to the overhang.

For SNP TSC1130902, the template DNA in lanes 1, 2, and 4 is homozygous for adenine at the variable site, as evidenced by a single higher molecular weight band migrating at about position 62 on the gel. The template DNA in lane 3 is heterozygous at the variable site, as indicated by the presence of two distinct bands. The lower band corresponds to molecules filled in with ddGTP at position 1 complementary to the overhang (the guanine allele). The higher molecular weight band corresponds to molecules filled in with ddGTP at position 3 complementary to the overhang (the adenine allele).

For SNP TSC0597888, the template DNA in lanes 1 and 4 was homozygous for cytosine at the variable site; the template DNA in lane 2 was heterozygous at the variable site, and the template DNA in lane 3 was homozygous for guanine. The expected fill-in reactions are diagrammed below:

Homozygous for cytosine:

	Allele 1	T	C	T	G	ATTC 3'
			G*	A	C	TAAG 5'
		4	3	2	1	Overhang position
	Allele 2	T	C	T	G	ATTC 3'
			G*	A	C	TAAG 5'
		4	3	2	1	Overhang position

Homozygous for guanine:

	Allele 1	T	C	T	C	ATTC 3'
					G*	TAAG 5'
		4	3	2	1	Overhang position
	Allele 2	T	C	T	C	ATTC 3'
					G*	TAAG 5'
		4	3	2	1	Overhang position

Heterozygous for guanine/cytosine:

	Allele 1	T	C	T	G	ATTC 3'
			G*	A	C	TAAG 5'
		4	3	2	1	Overhang position
	Allele 2	T	C	T	C	ATTC 3'
					G*	TAAG 5'
		4	3	2	1	Overhang position

Template DNA homozygous for guanine at the variable site displayed a single band, which corresponded to the DNA molecules filled in with ddGTP at position 1 complementary to the overhang. These DNA molecules were of lower molecular weight compared to the DNA molecules filled in with ddGTP at position 3 of the overhang (see lane 3 for SNP TSC0597888). The DNA molecules differed by two bases in molecular weight.

Template DNA homozygous for cytosine at the variable site displayed a single band, which corresponds to the DNA molecules filled in with ddGTP at position 3 complementary to the overhang. These DNA molecules migrated at a higher molecular weight than DNA molecules filled in with ddGTP at position 1 (see lanes 1 and 4 for SNP TSC0597888).

Template DNA heterozygous at the variable site displayed two bands; one band corresponded to the DNA molecules filled in with ddGTP at position 1 complementary to the overhang and was of lower molecular weight, and the second band corresponded to DNA molecules filled in with ddGTP at position 3 complementary to the overhang, and was of higher molecular weight (see lane 3 for SNP TSC0597888).

For SNP TSC0195492, the template DNA in lanes 1 and 3 was heterozygous at the variable site, which was demonstrated by the presence of two distinct bands. The template DNA in lane 2 was homozygous for guanine at the variable site. The template DNA in lane 4 was homozygous for cytosine. Only one band was seen in lane 4 for this SNP, and it had a higher molecular weight than the DNA molecules filled in with ddGTP at position 1 complementary to the overhang (compare lanes 2, 3 and 4).

The observed alleles for SNP TSC0607185 are reported as cytosine or thymidine. For consistency, the SNP consortium denotes the observed alleles as they appear in the sense strand www.snp.cshl.org/shpsearch.shtml; website active as of February 11, 2003). For this SNP, the second primer annealed from the locus of interest, which allowed the fill-in reaction to occur on the antisense strand after digestion with BsmF I.

The template DNA in lanes 1 and 3 was heterozygous; the template DNA in lane 2 was homozygous for thymidine, and the template DNA in lane 4 was homozygous for cytosine. The antisense strand was filled in with ddGTP, so the nucleotide on the sense strand corresponded to cytosine.

Molecular weight markers can be used to identify the positions of the expected bands. Alternatively, for each SNP analyzed, a known heterozygous sample can be used, which will identify precisely the position of the two expected bands.

As demonstrated in FIG. 11, one nucleotide labeled with one fluorescent dye can be used to determine the identity of a variable site including but not limited to SNPs and single nucleotide mutations. Typically, to determine if an individual is homozygous or heterozygous at a SNP site, multiple reactions are performed using one nucleotide labeled with one dye and a second nucleotide labeled with a second dye. However, this introduces problems in comparing results because the two dyes have different quantum coefficients. Even if different nucleotides are labeled with the same dye, the quantum coefficients are different. The use of a single nucleotide labeled with one dye eliminates any errors from the quantum coefficients of different dyes.

In this example, fluorescently labeled ddGTP was used. However, the method is applicable for a nucleotide tagged with any signal generating moiety including but not limited to radioactive molecule, fluorescent molecule, antibody, antibody fragment, hapten, carbohydrate, biotin, derivative of biotin, phosphorescent moiety, luminescent moiety, electrochemiluminescent moiety, chromatic moiety, and moiety having a detectable electron spin resonance, electrical capacitance, dielectric constant or electrical conductivity. In addition, labeled ddATP, ddTTP, or ddCTP can be used.

The above example used the third position complementary to the overhang as an indicator of the second allele. However, the second or fourth position of the overhang can be used as well (see Section on Incorporation of Nucleotides). Furthermore, the overhang was generated with the type IIS enzyme BsmF I; however any enzymes that cuts DNA at a distance from its binding site can be used including but not limited to the enzymes listed in Table I.

Also, in the above example, the nucleotide immediately preceding the SNP site was not a guanine on the strand that was filled in. This eliminated any effects of the alternative cutting properties of the type IIS restriction enzyme to be removed. For example, at SNP TSC0837969, the nucleotide from the SNP site on the sense strand was an adenine. If BsmF I displayed alternate cutting properties, the following overhangs would be generated for the adenine allele and the guanine allele:

G allele – 11/15 Cut 5' TTA

		3' AAT	T	C	A
	C				
	Overhang position		0	1	2
	3				
	G allele after fill-in	5' TTA	A	G*	
		3' AAT	T	C	A
	C				
	Overhang position		0	1	2
	3				
	A allele 11/15 Cut	5' TTA			
		3' AAT	T	T	A
	C				
	Overhang position		0	1	2
	3				
	A allele after fill-in	5' TTA	A	A	T
	G*				
		3' AAT	T	T	A
	C				
	Overhang position		0	1	2
	3				

For the guanine allele, the first position in the overhang would be filled in with dATP, which would allow the polymerase to incorporate ddGTP at position 2 complementary to the overhang. There would be no detectable difference between molecules cut at the 10/14 position or molecules cut at the 11/15 position.

For the adenine allele, the first position complementary to the overhang would be filled in with dATP, the second position would be filled in with dATP, the third position would be filled in with dTTP, and the fourth position would be filled in with ddGTP. There would be no difference in the molecular weights between molecules cut at 10/14 or

molecules cut at 11/15. The only differences would correspond to whether the DNA molecules contained an adenine at the variable site or a guanine at the variable site.

As seen in FIG. 11, positioning the annealing region of the first primer allows multiple SNPs to be analyzed in a single lane of a gel. Also, when using the same nucleotide with the same dye, a single fill-in reaction can be performed. In this example, 6 SNPs were analyzed in one lane. However, any number of SNPs including but not limited to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 30-40, 410-50, 510-60, 610-70, 710-80, 810-100, 1010-120, 1210-140, 1410-160, 1610-180, 1810-200, and greater than 200 can be analyzed in a single reaction.

Furthermore, one labeled nucleotide used to detect both alleles can be mixed with a second labeled nucleotide used to detect a different set of SNPs provided that neither of the nucleotides that are labeled occur immediately before the variable site (complementary to nucleotide at position 0 of the 11/15 cut) For example, suppose SNP X can be guanine or thymidine at the variable site and has the following 5' overhang generated after digestion with BsmF I:

SNP X 10/14	5' TTGAC				
G allele	3'AACTG	C	A	C	T
	Overhang position	1	2	3	4
SNP X 11/15	5' TTGA				
G allele	3'AACT	G	C	A	C
	Overhang position	0	1	2	3
SNP X 10/14	5' TTGAC				
T allele	3'AACTG	A	A	C	T
	Overhang position	1	2	3	4
SNP X 11/15	5' TTGA				
T allele	3'AACT	G	A	A	C
	Overhang position	0	1	2	3

After the fill-in reaction with labeled ddGTP, unlabeled dATP, dCTP, and dTTP, the following molecules would be generated:

	SNP X 10/14	5' TTGAC	G*			
	G allele	3'AACTG	C	A	C	T
	Overhang position		1	2	3	4
	SNP X 11/15	5' TTGA	C	G*		
	G allele	3'AACT	G	C	A	C
	Overhang position		0	1	2	3
	SNP X 10/14	5' TTGAC	T	T	G*	
	T allele	3'AACTG	A	A	C	T
	Overhang position		1	2	3	4
	SNP X 11/15	5' TTGA	C	T	T	G*
	T allele	3'AACT	G	A	A	C
	Overhang position		0	1	2	3

Now suppose SNP Y can be adenine or thymidine at the variable site, and has the following 5' overhangs generated after digestion with BsmF I.

	SNP Y 10/14	5' GTTT				
	A allele	3' CAAA	T	G	T	A
	Overhang position		1	2	3	4
	SNP Y 11/15	5' GTT				
	A allele	3' CAAA	T	G	T	
	Overhang position		0	1	2	3
	SNP Y 10/14	5' GTTT				
	T allele	3' CAAA	A	G	T	A

	Overhang position		1	2	3	4
	SNP Y 11/15	5' GTT				
	T allele	3' CAAA	A	G	T	
	Overhang position		0	1	2	3

After fill-in with labeled ddATP and unlabeled dCTP, dGTP, and dTTP, the following molecules would be generated:

	SNP Y 10/14	5' GTTT	A*			
	A allele	3' CAAA	T	G	T	A
	Overhang position		1	2	3	4
	SNP Y 11/15	5' GTT	T	A*		
	A allele	3' CAAA	T	G	T	
	Overhang position		0	1	2	3
	SNP Y 10/14	5' GTTT	T	C	A*	
	T allele	3' CAAA	A	G	T	A
	Overhang position		1	2	3	4
	SNP Y 11/15	5' GTT	T	T	C	A*
	T allele	3' CAAA	A	G	T	
	Overhang position		0	1	2	3

In this example, labeled ddGTP and labeled ddATP are used to determine the identity of both alleles of SNP X and SNP Y respectively. The nucleotide immediately preceding (the complementary nucleotide to position 0 of the overhang from the 11/15 cut SNP X is not guanine or adenine on the strand that is filled-in. Likewise, the nucleotide immediately preceding SNPY is not guanine or adenine on the strand that is filled-in. This allows the fill-in reaction for both SNPs to occur in a single reaction with labeled ddGTP, labeled ddATP, and unlabeled dCTP and dTTP. This reduces the number

of reactions that need to be performed and increases the number of SNPs that can be analyzed in one reaction.

The first primers for each SNP can be designed to anneal at different distances from the locus of interest, which allows the SNPs to migrate at different positions on the gel. For example, the first primer used to amplify SNP X can anneal at 30 bases from the locus of interest, and the first primer used to amplify SNP Y can anneal at 35 bases from the locus of interest. Also, the nucleotides can be labeled with fluorescent dyes that emit at spectrums that do not overlap. After running the gel, the gel can be scanned at one wavelength specific for one dye. Only those molecules labeled with that dye will emit a signal. The gel then can be scanned at the wavelength for the second dye. Only those molecules labeled with that dye will emit a signal. This method allows maximum compression for the number of SNPs that can be analyzed in a single reaction.

In this example, the nucleotide preceding the variable site on the strand that was filled-in was not adenine or guanine, and the nucleotide following the variable site can not be adenine or guanine on the sense strand. This method can work with any combination of labeled nucleotides, and the skilled artisan would understand which labeling reactions can be mixed and those that can not. For instance, if one SNP is labeled with thymidine and a second SNP is labeled with cytosine, the SNPs can be labeled in a single reaction if the nucleotide immediately preceding each variable site is not thymidine or cytosine on the sense strand and the nucleotide immediately after the variable site is not thymidine or cytosine on the sense strand.

This method allows the signals from one allele to be compared to the signal from a second allele without the added complexity of determining the degree of alternate cutting, or having to correct for the quantum coefficients of the dyes. This method is especially useful when trying to quantitate a ratio for one allele to another. For example, this method is useful for detecting chromosomal abnormalities. The ratio of alleles at a heterozygous site is expected to be about 1:1 (one A allele and one G allele). However, if an extra chromosome is present the ratio is expected to be about 1:2 (one A allele and 2 G alleles or 2 A alleles and 1 G allele). This method is especially useful when trying to detect fetal DNA in the presence of maternal DNA.

In addition, this method is useful for detecting two genetic signals in one sample. For example, this method can detect mutant cells in the presence of wild type cells (see Example 5). If a mutant cell contains a mutation in the DNA sequence of a particular

gene, this method can be used to detect both the mutant signal and the wild type signal. This method can be used to detect the mutant DNA sequence in the presence of the wild type DNA sequence. The ratio of mutant DNA to wild type DNA can be quantitated because a single nucleotide labeled with one signal generating moiety is used.

5

EXAMPLE 7

Non-invasive methods for the detection of various types of cancer have the potential to reduce morbidity and mortality from the disease. Several techniques for the
10 early detection of colorectal tumors have been developed including colonoscopy, barium enemas, and sigmoidoscopy; however the techniques are limited in use because they are invasive, which causes a low rate of patient compliance. Non-invasive genetic tests may be useful in identifying early stage colorectal tumors.

In 1991, researchers identified the Adenomatous Polyposis Coli gene (APC),
15 which plays a critical role in the formation of colorectal tumors (Kinzler *et al.*, Science 253:661-665, 1991). The APC gene resides on chromosome 5q21-22 and a total of 15 exons code for an RNA molecule of 8529 nucleotides, which produces a 300 Kd APC protein. The protein is expressed in numerous cell types and is essential for cell adhesion.

20 Mutations in the APC gene generally initiate colorectal neoplasia (Tsao, J. *et al.*, Am, J. Pathol. 145:531-534, 1994). Approximately 95% of the mutations in the APC gene result in nonsense/frameshift mutations. The most common mutations occur at codons 1061 and 1309; mutations at these codons account for 1/3 of all germline mutations. With regard to somatic mutations, 60% occur within codons 1286-1513,
25 which is about 10% of the coding sequence. This region is termed the mutation Cluster Region (MCR). Numerous types of mutations have been identified in the APC gene including nucleotide substitutions (see Table VII), splicing errors (see Table VIII), small deletions (see Table IX), small insertions (see Table X), small insertions/deletions (see Table XI), gross deletions (see Table XII), gross insertions (see Table XIII), and complex
30 rearrangements (see Table XIV).

Researchers have attempted to identify cells harboring mutations in the APC gene in stool samples (Traverso, G. *et al.*, New England Journal of Medicine, Vol 346:311-320, 2002). While APC mutations are found in nearly all tumors, about 1 in 250

cells in the stool sample has a mutation in the APC gene; most of the cells are normal cells that have been shed into the feces. Furthermore, human DNA represents about one-billionth of the total DNA found in stool samples; the majority of DNA is bacterial. The technique employed by Traverso *et al.* only detects mutations that result in a truncated protein.

As discussed above, numerous mutations in the APC gene have been implicated in the formation of colorectal tumors. Thus, a need still exists for a highly sensitive, non-invasive technique for the detection of colorectal tumors. Below, methods are described for detection of two mutations in the APC gene. However, any number of mutations can be analyzed using the methods described herein.

Preparation of Template DNA

The template DNA is purified from a sample containing colon cells including but not limited to a stool sample. The template DNA is purified using the procedures described by Ahlquist *et al.* (Gastroenterology, 119:1219-1227, 2000). If stool samples are frozen, the samples are thawed at room temperature, and homogenized with an Exactor stool shaker (Exact Laboratories, Maynard, Mass.) Following homogenization, a 4 gram stool equivalent of each sample is centrifuged at 2536 x g for 5 minutes. The samples are centrifuged a second time at 16, 500 x g for 10 minutes. Supernatants are incubated with 20 µl of RNase (0.5 mg per mililter) for 1 hour at 37°C. DNA is precipitated with 1/10 volume of 3 mol of sodium acetate per liter and an equal volume of isopropanol. The DNA is dissolved in 5 ml of TRIS-EDTA (0.01 mol of Tris per liter (pH 7.4) and 0.001 mole of EDTA per liter.

Design of Primers

To determine if a mutation resides at codon 1370, the following primers are used:

First primer:

5' GTGCAAAGGCCTGAATTCCCAGGCACAAAGCTGTTGAA 3'

Second primer:

5' TGAAGCGAACTAGGGACTCAGGTGGACTT

The first primer contains a biotin tag at the extreme 5' end, and the nucleotide sequence for the restriction enzyme EcoRI. The second primer contains the nucleotide sequence for the restriction enzyme BsmF I.

To determine if a small deletion exists at codon 1302, the following primers are used:

First primer:

5' GATTCCGTAAACGAATTCAGTTCATTATCATCTTTGTC 3'

Second primer:

5' CCATTGTTAAGCGGGACTTCTGCTATTTG 3'

The first primer has a biotin tag at the 5' end and contains a restriction enzyme recognition site for EcoRI. The second primer contains a restriction enzyme recognition site for BsmF I.

PCR Reaction

The loci of interest are amplified from the template genomic DNA using the polymerase chain reaction (PCR, U.S. Patent Nos. 4,683,195 and 4,683,202, incorporated herein by reference). The loci of interest are amplified in separate reaction tubes; they can also be amplified together in a single PCR reaction. For increased specificity, a "hot-start" PCR reaction is used, e.g. by using the HotStarTaq Master Mix Kit supplied by QIAGEN (catalog number 203443). The amount of template DNA and primer per reaction are optimized for each locus of interest but in this example, 40 ng of template human genomic DNA and 5 μM of each primer are used. Forty cycles of PCR are performed. The following PCR conditions are used:

(1) 95°C for 15 minutes and 15 seconds;
(2) 37°C for 30 seconds;
(3) 95°C for 30 seconds;
(4) 57°C for 30 seconds;
(5) 95°C for 30 seconds;
(6) 64°C for 30 seconds;
(7) 95°C for 30 seconds;

(8) Repeat steps 6 and 7 thirty nine (39) times;

(9) 72°C for 5 minutes.

In the first cycle of PCR, the annealing temperature is about the melting temperature of the 3′ annealing region of the second primers, which is 37°C. The annealing temperature in the second cycle of PCR is about the melting temperature of the 3' region, which anneals to the template DNA, of the first primer, which is 57°C. The annealing temperature in the third cycle of PCR is about the melting temperature of the entire sequence of the second primer, which is 64°C. The annealing temperature for the remaining cycles is 64°C. Escalating the annealing temperature from TM1 to TM2 to TM3 in the first three cycles of PCR greatly improves specificity. These annealing temperatures are representative, and the skilled artisan understands that the annealing temperatures for each cycle are dependent on the specific primers used.

The temperatures and times for denaturing, annealing, and extension, are optimized by trying various settings and using the parameters that yield the best results.

Purification of Fragment of Interest

The PCR products are separated from the genomic template DNA. Each PCR product is divided into four separate reaction wells of a Streptawell, transparent, High-Bind plate from Roche Diagnostics GmbH (catalog number 1 645 692, as listed in Roche Molecular Biochemicals, 2001 Biochemicals Catalog). The first primers contain a 5' biotin tag so the PCR products bound to the Streptavidin coated wells while the genomic template DNA does not. The streptavidin binding reaction is performed using a Thermomixer (Eppendorf) at 1000 rpm for 20 min. at 37°C. Each well is aspirated to remove unbound material, and washed three times with 1X PBS, with gentle mixing (Kandpal et al., Nucl. Acids Res. 18:1789-1795 (1990); Kaneoka et al., Biotechniques 10:30-34 (1991); Green et al., Nucl. Acids Res. 18:6163-6164 (1990)).

Alternatively, the PCR products are placed into a single well of a streptavidin plate to perform the nucleotide incorporation reaction in a single well.

Restriction Enzyme Digestion of Isolated Fragments

The purified PCR products are digested with the restriction enzyme BsmF I (New England Biolabs catalog number R0572S), which binds to the recognition site

incorporated into the PCR products from the second primer. The digests are performed in the Streptawells following the instructions supplied with the restriction enzyme. After digestion with the appropriate restriction enzyme, the wells are washed three times with PBS to remove the cleaved fragments.

Incorporation of Labeled Nucleotide

The restriction enzyme digest described above yields a DNA fragment with a 5' overhang, which contains the locus of interest and a 3' recessed end. The 5' overhang functions as a template allowing incorporation of a nucleotide or nucleotides in the presence of a DNA polymerase.

For each locus of interest, four separate fill in reactions are performed; each of the four reactions contains a different fluorescently labeled ddNTP (ddATP, ddTTP, ddGTP, or ddCTP). The following components are added to each fill in reaction: 1 μl of a fluorescently labeled ddNTP, 0.5 μl of unlabeled ddNTPs (40 μM), which contains all nucleotides except the nucleotide that is fluorescently labeled, 2 μl of 10X sequenase buffer, 0.25 μl of Sequenase, and water as needed for a 20μl reaction. The fill are performed in reactions at 40°C for 10 min. Non-fluorescently labeled ddNTP are purchased from Fermentas Inc. (Hanover, MD). All other labeling reagents are obtained from Amersham (Thermo Sequenase Dye Terminator Cycle Sequencing Core Kit, US 79565). In the presence of fluorescently labeled ddNTPs, the 3' recessed end is extended by one base, which corresponds to the locus of interest.

A mixture of labeled ddNTPs and unlabeled dNTPs also can be used for the fill-in reaction. The "fill in" conditions are as described above except that a mixture containing 40 μM unlabeled dNTPs, 1 μl fluorescently labeled ddATP, 1 μl fluorescently labeled ddTTP, 1 μl fluorescently labeled ddCTP, and 1 μl ddGTP are used. The fluorescent ddNTPs are obtained from Amersham (Thermo Sequenase Dye Terminator Cycle Sequencing Core Kit, US 79565; Amersham does not publish the concentrations of the fluorescent nucleotides). The locus of interest is digested with the restriction enzyme BsmF I, which generates a 5' overhang of four bases. If the first nucleotide incorporated is a labeled ddNTP, the 3' recessed end is filled in by one base, allowing detection of the locus of interest. However, if the first nucleotide incorporated is a dNTP, the polymerase continues to incorporate nucleotides until a ddNTP is filled in. For example, the first two nucleotides may be filled in with dNTPs, and the third nucleotide with a ddNTP, allowing

detection of the third nucleotide in the overhang. Thus, the sequence of the entire 5' overhang is determined, which increases the information obtained from each SNP or locus of interest. This type of fill in reaction is especially useful when detecting the presence of insertions, deletions, insertions and deletions, rearrangements, and translocations.

Alternatively, one nucleotide labeled with a single dye is used to determine the sequence of the locus of interest. See Example 6. This method eliminates any potential errors when using different dyes, which have different quantum coefficients.

After labeling, each Streptawell is rinsed with 1X PBS (100 µl) three times. The "filled in" DNA fragments are released from the Streptawells by digesting with the restriction enzyme EcoRI, according to the manufacturer's instructions that are supplied with the enzyme. The digestion is performed for 1 hour at 37 °C with shaking at 120 rpm.

Detection of the Locus of Interest

After release from the streptavidin matrix, the sample is loaded into a lane of a 36 cm 5% acrylamide (urea) gel (BioWhittaker Molecular Applications, Long Ranger Run Gel Packs, catalog number 50691). The sample is electrophoresed into the gel at 3000 volts for 3 min. The gel is run for 3 hours using a sequencing apparatus (Hoefer SQ3 Sequencer). The incorporated labeled nucleotide is detected by fluorescence.

To determine if any cells contain mutations at codon 1370 of the APC gene when separate fill-in reactions are performed, the lanes of the gel that correspond to the fill-in reaction for ddATP and ddTTP are analyzed. If only normal cells are present, the lane corresponding to the fill in reaction with ddATP is a bright signal. No signal is detected for the "fill-in" reaction with ddTTP. However, if the patient sample contains cells with mutations at codon 1370 of the APC gene, the lane corresponding to the fill in reaction with ddATP is a bright signal, and a signal is detected from the lane corresponding to the fill in reaction with ddTTP. The intensity of the signal from the lane corresponding to the fill in reaction with ddTTP is indicative of the number of mutant cells in the sample.

Alternatively, one labeled nucleotide is used to determine the sequence of the alleles at codon 1370 of the APC gene. At codon 1370, the normal sequence is AAA, which codes for the amino acid lysine. However, a nucleotide substitution has been identified at codon 1370, which is associated with colorectal tumors. Specifically, a

change from A to T (AAA-TAA) typically is found at codon 1370, which results in a stop codon. A single fill-in reaction is performed using labeled ddATP, and unlabeled dTTP, dCTP, and dGTP. A single nucleotide labeled with one fluorescent dye is used to determine the presence of both the normal and mutant DNA sequence that codes for codon 1370. The relevant DNA sequence is depicted below with the sequence corresponding to codon 1370 in bold:

5' CCCAAAAGTCCACCTGA
3' GGGTTTTCAGGTGGACT

After digest with BsmF I, the following overhang is produced:

5' CCC					
3' GGG	T	T	T	T	
Overhang position	1	2	3	4	

If the patient sample has no cells harboring a mutation at codon 1370, one signal is seen corresponding to incorporation of labeled ddATP.

5' CCC	**A***				
3' GGG	T	T	T	T	
Overhang position	1	2	3	4	

However, if the patient sample has cells with mutations at codon 1370 of the APC gene, one signal is seen, which corresponds to the normal sequence at codon 1370, and a second signal is seen, which corresponds to the mutant sequence at codon 1370. The signals clearly are identified as they differ in molecular weight.

Overhang of normal DNA sequence:	CCC					
	GGG	T	T	T	T	
Overhang position		1	2	3	4	

Normal DNA sequence after fill-in: CCC **A***

	GGG	T	T	T	T
Overhang position		1	2	3	4
Overhang of mutant DNA sequence:	CCC				
	GGG	A	T	T	T
Overhang position		1	2	3	4
Mutant DNA sequence after fill-in:	CCC	**T**	**A***		
	GGG	**A**	T	T	T
Overhang position		1	2	3	4

Two signals are seen when the mutant allele is present. The mutant DNA molecules are filled in one base after the wild type DNA molecules. The two signals are separated using any method that discriminates based on molecular weight. One labeled nucleotide (ddATP) is used to detect the presence of both the wild type DNA sequence and the mutant DNA sequence. This method of labeling reduces the number of reactions that need to be performed and allows accurate quantitation for the number of mutant cells in the patient sample. The number of mutant cells in the sample is used to determine patient prognosis, the degree and the severity of the disease. This method of labeling eliminates the complications associated with using different dyes, which have distinct quantum coefficients. This method of labeling also eliminates errors associated with pipetting reactions.

To determine if any cells contain mutations at codon 1302 of the APC gene when separate fill-in reactions are performed, the lanes of the gel that correspond to the fill-in reaction for ddTTP and ddCTP are analyzed. The normal DNA sequence is depicted below with sequence coding for codon 1302 in bold type-face.

Normal Sequence: 5' ACC**CTG**CAAATAGCAGAA
3' TGG**GAC**GTTTATCGTCTT

After digest, the following 5' overhang is produced:

5' ACCC

	3' TGGG	A	C	G	T
Overhang position		1	2	3	4

After the fill-in reaction, labeled ddTTP is incorporated.

	5' ACCC	**T***			
	3' TGGG	A	C	G	T
Overhang position		1	2	3	4

A deletion of a single base of the APC sequence, which typically codes for codon 1302, has been associated with colorectal tumors. The mutant DNA sequence is depicted below with the relevant sequence in bold:

Mutant Sequence: 5' ACCC**G**CAAATAGCAGAA
3' TGGG**C**GTTTATCGTCTT

After digest:

	5' ACC				
	3' TGG	G	C	G	T
Overhang position		1	2	3	4

After fill-in:

	5' ACC	**C***			
	3' TGG	G	C	G	T
Overhang position		1	2	3	4

If there are no mutations in the APC gene, signal is not detected for the fill in reaction with ddCTP*, but a bright signal is detected for the fill-in reaction with ddTTP*. However, if there are cells in the patient sample that have mutations in the APC gene, signals are seen for the fill-in reactions with ddCTP*and ddTTP*.

Alternatively, a single fill-in reaction is performed using a mixture containing unlabeled dNTPs, fluorescently labeled ddATP, fluorescently labeled ddTTP,

fluorescently labeled ddCTP, and fluorescently labeled ddGTP. If there is no deletion, labeled ddTTP is incorporated.

	5' ACCC	T*			
	3' TGGG	A	C	G	T
	Overhang position	1	2	3	4

However, if the T has been deleted, labeled ddCTP* is incorporated.

	5' ACCC*				
	3' TGGG	C	G	T	
	Overhang position	1	2	3	4

The two signals are separated by molecular weight because of the deletion of the thymidine nucleotide. If mutant cells are present, two signals are generated in the same lane but are separated by a single base pair (this principle is demonstrated in FIG 9D). The deletion causes a change in the molecular weight of the DNA fragments, which allows a single fill in reaction to be used to detect the presence of both normal and mutant cells.

In the above example, methods for the detection of a nucleotide substitution and a small deletion are described. However, the methods can be used for the detection of any type of mutation including but not limited to nucleotide substitutions (see Table VII), splicing errors (see Table VIII), small deletions (see Table IX), small insertions (see Table X), small insertions/deletions (see Table XI), gross deletions (see Table XII), gross insertions (see Table XIII), and complex rearrangements (see Table XIV).

In addition, the above-described methods are used for the detection of any type of disease including but not limited to those listed in Table IV. Furthermore, any type of mutant gene is detected using the inventions described herein including but not limited to the genes associated with the diseases listed in Table IV, BRCA1, BRCA2, MSH6, MSH2, MLH1, RET, PTEN, ATM, H-RAS, p53, ELAC2, CDH1, APC, AR, PMS2, MLH3, CYP1A1, GSTP1, GSTM1, AXIN2, CYP19, MET, NAT1, CDKN2A, NQO1, trc8, RAD51, PMS1, TGFBR2, VHL, MC4R, POMC, NROB2, UCP2, PCSK1, PPARG, ADRB2, UCP3, glur1, cart, SORBS1, LEP, LEPR, SIM1, TNF, IL-6, IL-1, IL-2, IL-3,

IL1A, TAP2, THPO, THRB, NBS1, RBM15, LIF, MPL, RUNX1, Her-2, glucocorticoid receptor, estrogen receptor, thyroid receptor, p21, p27, K-RAS, N-RAS, retinoblastoma protein, Wiskott-Aldrich (WAS) gene, Factor V Leiden, Factor II (prothrombin), methylene tetrahydrofolate reductase, cystic fibrosis, LDL receptor, HDL receptor, superoxide dismutase gene, SHOX gene, genes involved in nitric oxide regulation, genes involved in cell cycle regulation, tumor suppressor genes, oncogenes, genes associated with neurodegeneration, genes associated with obesity, . Abbreviations correspond to the proteins as listed on the Human Gene Mutation Database, which is incorporated herein by reference www.archive.uwcm.ac.uk./uwcm; website address active as of February 12, 2003).

The above-example demonstrates the detection of mutant cells and mutant alleles from a fecal sample. However, the methods described herein are used for detection of mutant cells from any biological sample including but not limited to blood sample, serum sample, plasma sample, urine sample, spinal fluid, lymphatic fluid, semen, vaginal secretion, ascitic fluid, saliva, mucosa secretion, peritoneal fluid, fecal sample, body exudates, breast fluid, lung aspirates, cells, tissues, individual cells or extracts of the such sources that contain the nucleic acid of the same, and subcellular structures such as mitochondria or chloroplasts. In addition, the methods described herein are used for the detection of mutant cells and mutated DNA from any number of nucleic acid containing sources including but not limited to forensic, food, archeological, agricultural or inorganic samples.

The above example is directed to detection of mutations in the APC gene. However, the inventions described herein are used for the detection of mutations in any gene that is associated with or predisposes to disease (see Table XV).

For example, hypermethylation of the glutathione S-transferase P1 (GSTP1) promoter is the most common DNA alteration in prostrate cancer. The methylation state of the promoter is determined using sodium bisulfite and the methods described herein.

Treatment with sodium bisulfite converts unmethylated cytosine residues into uracil, and leaving the methylated cytosines unchanged. Using the methods described herein, a first and second primer are designed to amplify the regions of the GSTP1 promoter that are often methylated. Below, a region of the GSTP1 promoter is shown prior to sodium bisulfite treatment:

Before Sodium Bisulfite treatment:

5' ACCGCTACA

3' TGGCGATCA

Below, a region of the GSTP1 promoter is shown after sodium bisulfite treatment, PCR amplification, and digestion with the type IIS restriction enzyme BsmF I:

Unmethylated					
	5' ACC				
	3' TGG	**U**	G	A	T
Overhang position		1	2	3	4
Methylated					
	5' ACC				
	3' TGG	**C**	G	A	T
Overhang position		1	2	3	4

Labeled ddATP, unlabeled dCTP, dGTP, and dTTP are used to fill-in the 5' overhangs. The following molecules are generated:

Unmethylated					
	5' ACC	**A***			
	3' TGG	**U**	G	A	T
Overhang position		1	2	3	4
Methylated					
	5' ACC	G	C	T	**A***
	3' TGG	**C**	G	A	T
Overhang position		1	2	3	4

Two signals are seen; one corresponds to DNA molecules filled in with ddATP at position one complementary to the overhang (unmethylated), and the other corresponds to the DNA molecules filled in with ddATP at position 4 complementary to the overhang (methylated). The two signals are separated based on molecular weight. Alternatively,

the fill-in reactions are performed in separate reactions using labeled ddGTP in one reaction and labeled ddATP in another reaction.

The methods described herein are used to screen for prostate cancer and also to monitor the progression and severity of the disease. The use of a single nucleotide to detect both the methylated and unmethylated sequences allows accurate quantitation and provides a high level of sensitivity for the methylated sequences, which is a useful tool for earlier detection of the disease.

The information contained in Tables VII-XIV was obtained from the Human Gene Mutation Database. With the information provided herein, the skilled artisan will understand how to apply these methods for determining the sequence of the alleles for any gene. A large number of genes and there associated mutations can be found at the following website: www.archive.uwcm.ac.uk./uwcm.

TABLE VII: NUCLEOTIDE SUBSTITUTIONS

Codon	**Nucleotide**	**Amino acid**	**Phenotype**
99	CGG-TGG	Arg-Trp	Adenomatous polyposis coli
121	AGA-TGA	Arg-Term	Adenomatous polyposis coli
157	TGG-TAG	Trp-Term	Adenomatous polyposis coli
159	TAC-TAG	Tyr-Term	Adenomatous polyposis coli
163	CAG-TAG	Gln-Term	Adenomatous polyposis coli
168	AGA-TGA	Arg-Term	Adenomatous polyposis coli
171	AGT-ATT	Ser-Ile	Adenomatous polyposis coli
181	CAA-TAA	Gln-Term	Adenomatous polyposis coli
190	GAA-TAA	Glu-Term	Adenomatous polyposis coli
202	GAA-TAA	Glu-Term	Adenomatous polyposis coli
208	CAG-CGG	Gln-Arg	Adenomatous polyposis coli
208	CAG-TAG	Gln-Term	Adenomatous polyposis coli
213	CGA-TGA	Arg-Term	Adenomatous polyposis coli
215	CAG-TAG	Gln-Term	Adenomatous polyposis coli
216	CGA-TGA	Arg-Term	Adenomatous polyposis coli
232	CGA-TGA	Arg-Term	Adenomatous polyposis coli
233	CAG-TAG	Gln-Term	Adenomatous polyposis coli

247	CAG-TAG	Gln-Term	Adenomatous polyposis coli
267	GGA-TGA	Gly-Term	Adenomatous polyposis coli
278	CAG-TAG	Gln-Term	Adenomatous polyposis coli
280	TCA-TGA	Ser-Term	Adenomatous polyposis coli
280	TCA-TAA	Ser-Term	Adenomatous polyposis coli
283	CGA-TGA	Arg-Term	Adenomatous polyposis coli
302	CGA-TGA	Arg-Term	Adenomatous polyposis coli
332	CGA-TGA	Arg-Term	Adenomatous polyposis coli
358	CAG-TAG	Gln-Term	Adenomatous polyposis coli
405	CGA-TGA	Arg-Term	Adenomatous polyposis coli
414	CGC-TGC	Arg-Cys	Adenomatous polyposis coli
422	GAG-TAG	Glu-Term	Adenomatous polyposis coli
423	TGG-TAG	Trp-Term	Adenomatous polyposis coli
424	CAG-TAG	Gln-Term	Adenomatous polyposis coli
433	CAG-TAG	Gln-Term	Adenomatous polyposis coli
443	GAA-TAA	Glu-Term	Adenomatous polyposis coli
457	TCA-TAA	Ser-Term	Adenomatous polyposis coli
473	CAG-TAG	Gln-Term	Adenomatous polyposis coli
486	TAC-TAG	Tyr-Term	Adenomatous polyposis coli
499	CGA-TGA	Arg-Term	Adenomatous polyposis coli
500	TAT-TAG	Tyr-Term	Adenomatous polyposis coli
541	CAG-TAG	Gln-Term	Adenomatous polyposis coli
553	TGG-TAG	Trp-Term	Adenomatous polyposis coli
554	CGA-TGA	Arg-Term	Adenomatous polyposis coli
564	CGA-TGA	Arg-Term	Adenomatous polyposis coli
577	TTA-TAA	Leu-Term	Adenomatous polyposis coli
586	AAA-TAA	Lys-Term	Adenomatous polyposis coli
592	TTA-TGA	Leu-Term	Adenomatous polyposis coli
593	TGG-TAG	Trp-Term	Adenomatous polyposis coli
593	TGG-TGA	Trp-Term	Adenomatous polyposis coli
622	TAC-TAA	Tyr-Term	Adenomatous polyposis coli

625	CAG-TAG	Gln-Term	Adenomatous polyposis coli
629	TTA-TAA	Leu-Term	Adenomatous polyposis coli
650	GAG-TAG	Glu-Term	Adenomatous polyposis coli
684	TTG-TAG	Leu-Term	Adenomatous polyposis coli
685	TGG-TGA	Trp-Term	Adenomatous polyposis coli
695	CAG-TAG	Gln-Term	Adenomatous polyposis coli
699	TGG-TGA	Trp-Term	Adenomatous polyposis coli
699	TGG-TAG	Trp-Term	Adenomatous polyposis coli
713	TCA-TGA	Ser-Term	Adenomatous polyposis coli
722	AGT-GGT	Ser-Gly	Adenomatous polyposis coli
747	TCA-TGA	Ser-Term	Adenomatous polyposis coli
764	TTA-TAA	Leu-Term	Adenomatous polyposis coli
784	TCT-ACT	Ser-Thr	Adenomatous polyposis coli
805	CGA-TGA	Arg-Term	Adenomatous polyposis coli
811	TCA-TGA	Ser-Term	Adenomatous polyposis coli
848	AAA-TAA	Lys-Term	Adenomatous polyposis coli
876	CGA-TGA	Arg-Term	Adenomatous polyposis coli
879	CAG-TAG	Gln-Term	Adenomatous polyposis coli
893	GAA-TAA	Glu-Term	Adenomatous polyposis coli
932	TCA-TAA	Ser-Term	Adenomatous polyposis coli
932	TCA-TGA	Ser-Term	Adenomatous polyposis coli
935	TAC-TAG	Tyr-Term	Adenomatous polyposis coli
935	TAC-TAA	Tyr-Term	Adenomatous polyposis coli
995	TGC-TGA	Cys-Term	Adenomatous polyposis coli
997	TAT-TAG	Tyr-Term	Adenomatous polyposis coli
999	CAA-TAA	Gln-Term	Adenomatous polyposis coli
1000	TAC-TAA	Tyr-Term	Adenomatous polyposis coli
1020	GAA-TAA	Glu-Term	Adenomatous polyposis coli
1032	TCA-TAA	Ser-Term	Adenomatous polyposis coli
1041	CAA-TAA	Gln-Term	Adenomatous polyposis coli
1044	TCA-TAA	Ser-Term	Adenomatous polyposis coli

1045	CAG-TAG	Gln-Term	Adenomatous polyposis coli
1049	TGG-TGA	Trp-Term	Adenomatous polyposis coli
1067	CAA-TAA	Gln-Term	Adenomatous polyposis coli
1071	CAA-TAA	Gln-Term	Adenomatous polyposis coli
1075	TAT-TAA	Tyr-Term	Adenomatous polyposis coli
1075	TAT-TAG	Tyr-Term	Adenomatous polyposis coli
1102	TAC-TAG	Tyr-Term	Adenomatous polyposis coli
1110	TCA-TGA	Ser-Term	Adenomatous polyposis coli
1114	CGA-TGA	Arg-Term	Adenomatous polyposis coli
1123	CAA-TAA	Gln-Term	Adenomatous polyposis coli
1135	TAT-TAG	Tyr-Term	Adenomatous polyposis coli
1152	CAG-TAG	Gln-Term	Adenomatous polyposis coli
1155	GAA-TAA	Glu-Term	Adenomatous polyposis coli
1168	GAA-TAA	Glu-Term	Adenomatous polyposis coli
1175	CAG-TAG	Gln-Term	Adenomatous polyposis coli
1176	CCT-CTT	Pro-Leu	Adenomatous polyposis coli
1184	GCC-CCC	Ala-Pro	Adenomatous polyposis coli
1193	CAG-TAG	Gln-Term	Adenomatous polyposis coli
1194	TCA-TGA	Ser-Term	Adenomatous polyposis coli
1198	TCA-TGA	Ser-Term	Adenomatous polyposis coli
1201	TCA-TGA	Ser-Term	Adenomatous polyposis coli
1228	CAG-TAG	Gln-Term	Adenomatous polyposis coli
1230	CAG-TAG	Gln-Term	Adenomatous polyposis coli
1244	CAA-TAA	Gln-Term	Adenomatous polyposis coli
1249	TGC-TGA	Cys-Term	Adenomatous polyposis coli
1256	CAA-TAA	Gln-Term	Adenomatous polyposis coli
1262	TAT-TAA	Tyr-Term	Adenomatous polyposis coli
1270	TGT-TGA	Cys-Term	Adenomatous polyposis coli
1276	TCA-TGA	Ser-Term	Adenomatous polyposis coli
1278	TCA-TAA	Ser-Term	Adenomatous polyposis coli
1286	GAA-TAA	Glu-Term	Adenomatous polyposis coli

1289	TGT-TGA	Cys-Term	Adenomatous polyposis coli
1294	CAG-TAG	Gln-Term	Adenomatous polyposis coli
1307	ATA-AAA	Ile-Lys	Colorectal cancer, predisposition to, association
1309	GAA-TAA	Glu-Term	Adenomatous polyposis coli
1317	GAA-CAA	Glu-Gln	Colorectal cancer, predisposition to
1328	CAG-TAG	Gln-Term	Adenomatous polyposis coli
1338	CAG-TAG	Gln-Term	Adenomatous polyposis coli
1342	TTA-TAA	Leu-Term	Adenomatous polyposis coli
1342	TTA-TGA	Leu-Term	Adenomatous polyposis coli
1348	AGG-TGG	Arg-Trp	Adenomatous polyposis coli
1357	GGA-TGA	Gly-Term	Adenomatous polyposis coli
1367	CAG-TAG	Gln-Term	Adenomatous polyposis coli
1370	AAA-TAA	Lys-Term	Adenomatous polyposis coli
1392	TCA-TAA	Ser-Term	Adenomatous polyposis coli
1392	TCA-TGA	Ser-Term	Adenomatous polyposis coli
1397	GAG-TAG	Glu-Term	Adenomatous polyposis coli
1449	AAG-TAG	Lys-Term	Adenomatous polyposis coli
1450	CGA-TGA	Arg-Term	Adenomatous polyposis coli
1451	GAA-TAA	Glu-Term	Adenomatous polyposis coli
1503	TCA-TAA	Ser-Term	Adenomatous polyposis coli
1517	CAG-TAG	Gln-Term	Adenomatous polyposis coli
1529	CAG-TAG	Gln-Term	Adenomatous polyposis coli
1539	TCA-TAA	Ser-Term	Adenomatous polyposis coli
1541	CAG-TAG	Gln-Term	Adenomatous polyposis coli
1564	TTA-TAA	Leu-Term	Adenomatous polyposis coli
1567	TCA-TGA	Ser-Term	Adenomatous polyposis coli
1640	CGG-TGG	Arg-Trp	Adenomatous polyposis coli
1693	GAA-TAA	Glu-Term	Adenomatous polyposis coli
1822	GAC-GTC	Asp-Val	Adenomatous polyposis coli, association with ?
2038	CTG-GTG	Leu-Val	Adenomatous polyposis coli
2040	CAG-TAG	Gln-Term	Adenomatous polyposis coli

2566	AGA-AAA	Arg-Lys	Adenomatous polyposis coli
2621	TCT-TGT	Ser-Cys	Adenomatous polyposis coli
2839	CTT-TTT	Leu-Phe	Adenomatous polyposis coli

TABLE VIII: NUCLEOTIDE SUBSTITUTIONS

Donor/ Acceptor	**Relative location**	**Substitution**	**Phenotype**
ds	-1	G-C	Adenomatous polyposis coli
as	-1	G-A	Adenomatous polyposis coli
as	-1	G-C	Adenomatous polyposis coli
ds	+2	T-A	Adenomatous polyposis coli
as	-1	G-C	Adenomatous polyposis coli
as	-1	G-T	Adenomatous polyposis coli
as	-1	G-A	Adenomatous polyposis coli
as	-2	A-C	Adenomatous polyposis coli
as	-5	A-G	Adenomatous polyposis coli
ds	+3	A-C	Adenomatous polyposis coli
as	-1	G-A	Adenomatous polyposis coli
ds	+1	G-A	Adenomatous polyposis coli
as	-1	G-T	Adenomatous polyposis coli
ds	+1	G-A	Adenomatous polyposis coli
as	-1	G-A	Adenomatous polyposis coli
ds	+1	G-A	Adenomatous polyposis coli
ds	+3	A-G	Adenomatous polyposis coli
ds	+5	G-T	Adenomatous polyposis coli
as	-1	G-A	Adenomatous polyposis coli
as	-6	A-G	Adenomatous polyposis coli
as	-5	A-G	Adenomatous polyposis coli
as	-2	A-G	Adenomatous polyposis coli
ds	+2	T-C	Adenomatous polyposis coli
as	-2	A-G	Adenomatous polyposis coli

ds	+1	G-A	Adenomatous polyposis coli
ds	+1	G-T	Adenomatous polyposis coli
ds	+2	T-G	Adenomatous polyposis coli

TABLE IX: APC SMALL DELETIONS

Bold letters indicate the codon. Undercase letters represent the deletion. Where deletions extend beyond the coding region, other positional information is provided. For example, the abbreviation 5' UTR represents 5' untranslated region, and the abbreviation E6I6 denotes exon 6/intron 6 boundary.

Location/ codon	**Deletion**	**Phenotype**
77	**TTA**gataGCAGTAATTT	Adenomatous polyposis coli
97	**GGA**AGccgggaagGATCTGTATC	Adenomatous polyposis coli
138	**GAG**AaAGAGAG_E3I3_GTAA	Adenomatous polyposis coli
139	**AAA**GAgag_E3I3_Gtaacttttct	Thyroid cancer
139	**AAA**Gagag_E3I3_GTAACTTTTC	Adenomatous polyposis coli
142	TTTTAAAAAAaAAAAATAG_I3E4_**GTCA**	Adenomatous polyposis coli
144	AAAATAG_I3E4_GTCatTG**CTT**CTTGC	Adenomatous polyposis coli
149	**GACA**aaGAAGAAAAGG	Adenomatous polyposis coli
149	**GACA**AagaaGAAAAGGAAA	Adenomatous polyposis coli
155	AGGAA^**AAA**GActggtATTACGCTCA	Adenomatous polyposis coli

169	AAAAGA^**ATA**GatagTCTTCCTTTA	Adenomatous polyposis coli
172	AGATAGT^**CTT**cCTTTAACTGA	Adenomatous polyposis coli
179	**TCC**TTacaaACAGATATGA	Adenomatous polyposis coli
185	**ACC**aGAAGGCAATT	Adenomatous polyposis coli
196	**ATC**AGagTTGCGATGGA	Adenomatous polyposis coli
213	**CGA**GCaCAG_E5I5_GTAAGTT	Adenomatous polyposis coli
298	**CAC**tcTGCACCTCGA	Adenomatous polyposis coli
329	**GAT**aTGTCGCGAAC	Adenomatous polyposis coli
365	**AAA**GActCTGTATTGTT	Adenomatous polyposis coli
397	**GAC**aaGAGAGGCAGG	Adenomatous polyposis coli
427	**CAT**GAacCAGGCATGGA	Adenomatous polyposis coli
428	**GAA**CCaGGCATGGACC	Adenomatous polyposis coli
436	**AAT**CCaa_E9I9_gTATGTTCTCT	Adenomatous polyposis coli
440	**GCT**CCtGTTGAACATC	Adenomatous polyposis coli
455	**AAA**CTtTCATTTGATG	Adenomatous polyposis coli
455	**AAA**CtttcaTTTGATGAAG	Adenomatous polyposis coli

472	CTAcAGGCCATTGC	Adenomatous polyposis coli
472	TAAATTAG_I10E11_GGgGACTACAGGC	Adenomatous polyposis coli
478	TTATtGCAAGTGGAC	Adenomatous polyposis coli
486	TACGgGCTTACTAAT	Adenomatous polyposis coli
494	AGTATtACACTAAGAC	Adenomatous polyposis coli
495	ATTACacTAAGACGATA	Adenomatous polyposis coli
497	CTAaGACGATATGC	Adenomatous polyposis coli
520	TGCTCtaTGAAAGGCTG	Adenomatous polyposis coli
526	ATGAGagcacttgtgGCCCAACTAA	Adenomatous polyposis coli
539	GACTTaCAGCAG_E12I12_GTAC	Adenomatous polyposis coli
560	AAAAAgaCGTTGCGAGA	Adenomatous polyposis coli
566	GTTGgaagtGTGAAAGCAT	Adenomatous polyposis coli
570	AAAGCaTTGATGGAAT	Adenomatous polyposis coli
577	TTAGaagtTAAAAAG_E13I13_GTA	Adenomatous polyposis coli
584	ACCCTcAAAAGCGTAT	Adenomatous polyposis coli
591	GCCTtATGGAATTTG	Adenomatous polyposis coli

608	GCTgTAGATGGTGC	Adenomatous polyposis coli
617	GTTggcactcttacttaccGGAGCCAGAC	Adenomatous polyposis coli
620	CTTACttacCGGAGCCAGA	Adenomatous polyposis coli
621	ACTTaCCGGAGCCAG	Adenomatous polyposis coli
624	AGCcaGACAAACACT	Adenomatous polyposis coli
624	AGCCagacAAACACTTTA	Adenomatous polyposis coli
626	ACAaacaCTTTAGCCAT	Adenomatous polyposis coli
629	TTAGCcATTATTGAAA	Adenomatous polyposis coli
635	GGAGgTGGGATATTA	Adenomatous polyposis coli
638	ATATtACGGAATGTG	Adenomatous polyposis coli
639	TTACGgAATGTGTCCA	Adenomatous polyposis coli
657	AGAgaGAACAACTGT	Adenomatous polyposis coli
659	TATTTCAG_I14E15_GCaaatcctaagagagAACAACTGTC	Adenomatous polyposis coli
660	AACTgtCTACAAACTT	Adenomatous polyposis coli
665	TTAttACAACACTTA	Adenomatous polyposis coli
668	CACttAAAATCTCAT	Adenomatous polyposis coli

673	AGTttgacaatagtCAGTAATGCA	Adenomatous polyposis coli
768	CACTTaTCAGAAACTT	Adenomatous polyposis coli
769	TTATcAGAAACTTTT	Adenomatous polyposis coli
770	TCAGAaACTTTTGACA	Adenomatous polyposis coli
780	AGTCcCAAGGCATCT	Adenomatous polyposis coli
792	AAGCaAAGTCTCTAT	Adenomatous polyposis coli
792	AAGCAaaGTCTCTATGG	Adenomatous polyposis coli
793	CAAAgTCTCTATGGT	Adenomatous polyposis coli
798	GATTatGTTTTTGACA	Adenomatous polyposis coli
802	GACACcaatcgacatGATGATAATA	Adenomatous polyposis coli
805	CGACatGATGATAATA	Adenomatous polyposis coli
811	TCAGacaaTTTTAATACT	Adenomatous polyposis coli
825	TATtTGAATACTAC	Adenomatous polyposis coli
827	AATAcTACAGTGTTA	Adenomatous polyposis coli
830	GTGTTacccagctcctctTCATCAAGAG	Adenomatous polyposis coli
833	AGCTCcTCTTCATCAA	Adenomatous polyposis coli

836	TCATcAAGAGGAAGC	Adenomatous polyposis coli
848	AAAGAtaGAAGTTTGGA	Adenomatous polyposis coli
848	AAAGatagaagTTTGGAGAGA	Adenomatous polyposis coli
855	GAACgCGGAATTGGT	Adenomatous polyposis coli
856	CGCGgaattGGTCTAGGCA	Adenomatous polyposis coli
856	CGCGgAATTGGTCTA	Adenomatous polyposis coli
879	CAGaTCTCCACCAC	Adenomatous polyposis coli
902	GAAGAcagaAGTTCTGGGT	Adenomatous polyposis coli
907	GGGTcTACCACTGAA	Adenomatous polyposis coli
915	GTGACaGATGAGAGAA	Adenomatous polyposis coli
929	CATACacatTCAAACACTT	Adenomatous polyposis coli
930	ACACAttcaAACACTTACA	Adenomatous polyposis coli
931	CATtCAAACACTTA	Adenomatous polyposis coli
931	CATTcAAACACTTAC	Adenomatous polyposis coli
933	AACacttACAATTTCAC	Adenomatous polyposis coli
935	TACAatttcactAAGTCGGAAA	Adenomatous polyposis coli

937	TTCActaaGTCGGAAAAT	Adenomatous polyposis coli
939	AAGtcggAAAATTCAAA	Adenomatous polyposis coli
946	ACATgTTCTATGCCT	Adenomatous polyposis coli
954	TTAGaaTACAAGAGAT	Adenomatous polyposis coli
961	AATgATAGTTTAAA	Adenomatous polyposis coli
963	AGTTTaAATAGTGTCA	Adenomatous polyposis coli
964	TTAaataGTGTCAGTAG	Adenomatous polyposis coli
973	TATGgTAAAAGAGGT	Adenomatous polyposis coli
974	GGTAAaAGAGGTCAAA	Adenomatous polyposis coli
975	AAAAgaGGTCAAATGA	Thyroid cancer
992	AGTAAgTTTTGCAGTT	Thyroid cancer
993	AAGttttgcagttaTGGTCAATAC	Adenomatous polyposis coli
999	CAAtacccagCCGACCTAGC	Adenomatous polyposis coli
1023	ACACcAATAAATTAT	Adenomatous polyposis coli
1030	AAAtATTCAGATGA	Adenomatous polyposis coli
1032	TCAGatgagCAGTTGAACT	Adenomatous polyposis coli
1033	GATGaGCAGTTGAAC	Adenomatous polyposis coli

1049	TGGGcAAGACCCAAA	Adenomatous polyposis coli
1054	CACAtaataGAAGATGAAA	Adenomatous polyposis coli
1055	ATAAtagaaGATGAAATAA	Adenomatous polyposis coli
1056	ATAGAaGATGAAATAA	Adenomatous polyposis coli
1060	ATAAAacaaaGTGAGCAAAG	Adenomatous polyposis coli
1061	AAAcaaaGTGAGCAAAG	Adenomatous polyposis coli
1061	AAACaaAGTGAGCAAA	Adenomatous polyposis coli
1062	CAAAgtgaGCAAAGACAA	Adenomatous polyposis coli
1065	CAAAGacAATCAAGGAA	Adenomatous polyposis coli
1067	CAAtcaaGGAATCAAAG	Adenomatous polyposis coli
1071	CAAAgtACAACTTATC	Adenomatous polyposis coli
1079	ACTGagAGCACTGATG	Adenomatous polyposis coli
1082	ACTGAtgATAAACACCT	Adenomatous polyposis coli
1084	GATaaacACCTCAAGTT	Adenomatous polyposis coli
1086	CACCtcAAGTTCCAAC	Adenomatous polyposis coli
1093	TTTGgACAGCAGGAA	Adenomatous polyposis coli

1098	TGTgtTTCTCCATAC	Adenomatous polyposis coli
1105	CGGgGAGCCAATGG	Thyroid cancer
1110	TCAGAaACAAATCGAG	Adenomatous polyposis coli
1121	ATTAAtcaaAATGTAAGCC	Adenomatous polyposis coli
1131	CAAgAAGATGACTA	Adenomatous polyposis coli
1134	GACTAtGAAGATGATA	Adenomatous polyposis coli
1137	GATgataaGCCTACCAAT	Adenomatous polyposis coli
1146	CGTTAcTCTGAAGAAG	Adenomatous polyposis coli
1154	GAAGaagaaGAGAGACCAA	Adenomatous polyposis coli
1155	GAAGaagaGAGACCAACA	Adenomatous polyposis coli
1156	GAAgagaGACCAACAAA	Adenomatous polyposis coli
1168	GAAgagaaACGTCATGTG	Adenomatous polyposis coli
1178	GATTAtagtttaAAATATGCCA	Adenomatous polyposis coli
1181	TTAAaATATGCCACA	Adenomatous polyposis coli
1184	GCCacagaTATTCCTTCA	Adenomatous polyposis coli
1185	ACAgaTATTCCTTCA	Adenomatous polyposis coli
1190	TCACAgAAACAGTCAT	Adenomatous

		polyposis coli
1192	AAAcaGTCATTTTCA	Adenomatous polyposis coli
1198	TCAaaGAGTTCATCT	Adenomatous polyposis coli
1207	AAAAcCGAACATATG	Adenomatous polyposis coli
1208	ACCgaacATATGTCTTC	Adenomatous polyposis coli
1210	CATatGTCTTCAAGC	Adenomatous polyposis coli
1233	CCAAGtTCTGCACAGA	Adenomatous polyposis coli
1249	TGCAaaGTTTCTTCTA	Adenomatous polyposis coli
1259	ATAcaGACTTATTGT	Adenomatous polyposis coli
1260	CAGACttATTGTGTAGA	Adenomatous polyposis coli
1268	CCAaTATGTTTTTC	Adenomatous polyposis coli
1275	AGTtCATTATCATC	Adenomatous polyposis coli
1294	CAGGAaGCAGATTCTG	Adenomatous polyposis coli
1301	ACCCtGCAAATAGCA	Adenomatous polyposis coli
1306	GAAAtaaaAGAAAAGATT	Adenomatous polyposis coli
1307	ATAaAAGAAAAGAT	Adenomatous polyposis coli
1308	AAAgaaaAGATTGGAAC	Adenomatous

		polyposis coli
1308	AAAGAaaagaTTGGAACTAG	Adenomatous polyposis coli
1318	GATCcTGTGAGCGAA	Adenomatous polyposis coli
1320	GTGAGcGAAGTTCCAG	Adenomatous polyposis coli
1323	GTTCcAGCAGTGTCA	Adenomatous polyposis coli
1329	CACCctagaaccAAATCCAGCA	Adenomatous polyposis coli
1336	AGACtgCAGGGTTCTA	Adenomatous polyposis coli
1338	CAGgGTTCTAGTTT	Adenomatous polyposis coli
1340	TCTAgTTTATCTTCA	Adenomatous polyposis coli
1342	TTATcTTCAGAATCA	Adenomatous polyposis coli
1352	GTTgAATTTTCTTC	Adenomatous polyposis coli
1361	CCCTcCAAAAGTGGT	Adenomatous polyposis coli
1364	AGTggtgCTCAGACACC	Adenomatous polyposis coli
1371	AGTCCacCTGAACACTA	Adenomatous polyposis coli
1372	CCACCtGAACACTATG	Adenomatous polyposis coli
1376	TATGttCAGGAGACCC	Adenomatous polyposis coli
1394	GATAgtTTTGAGAGTC	Adenomatous

		polyposis coli
1401	ATTGCcAGCTCCGTTC	Adenomatous polyposis coli
1415	AGTGGcATTATAAGCC	Adenomatous polyposis coli
1426	AGCCcTGGACAAACC	Adenomatous polyposis coli
1427	CCTGGaCAAACCATGC	Adenomatous polyposis coli
1431	ATGCcACCAAGCAGA	Adenomatous polyposis coli
1454	AAAAAtAAAGCACCTA	Adenomatous polyposis coli
1461	GAAaAGAGAGAGAG	Adenomatous polyposis coli
1463	AGAgagaGTGGACCTAA	Adenomatous polyposis coli
1464	GAGAgTGGACCTAAG	Adenomatous polyposis coli
1464	GAGAgtGGACCTAAGC	Adenomatous polyposis coli
1464	GAGagTGGACCTAAG	Adenomatous polyposis coli
1492	GCCaCGGAAAGTAC	Adenomatous polyposis coli
1493	ACGGAaAGTACTCCAG	Adenomatous polyposis coli
1497	CCAgATGGATTTTC	Adenomatous polyposis coli
1503	TCAtccaGCCTGAGTGC	Adenomatous polyposis coli
1522	TTAagaataaTGCCTCCAGT	Adenomatous

		polyposis coli
1536	GAAACagAATCAGAGCA	Adenomatous polyposis coli
1545	TCAAAtgaaaACCAAGAGAA	Adenomatous polyposis coli
1547	GAAaACCAAGAGAA	Adenomatous polyposis coli
1550	GAGAaagaGGCAGAAAAA	Adenomatous polyposis coli
1577	GAATgtATTATTTCTG	Adenomatous polyposis coli
1594	CCAGCcCAGACTGCTT	Adenomatous polyposis coli
1596	CAGACtGCTTCAAAAT	Adenomatous polyposis coli
1823	TTCAaTGATAAGCTC	Adenomatous polyposis coli
1859	AATGAttctTTGAGTTCTC	Adenomatous polyposis coli
1941	CCAGAcagaGGGGCAGCAA	Desmoid tumours
1957	GAAaATACTCCAGT	Adenomatous polyposis coli
1980	AACaATAAAGAAAA	Adenomatous polyposis coli
1985	GAACCtATCAAAGAGA	Adenomatous polyposis coli
1986	CCTaTCAAAGAGAC	Adenomatous polyposis coli
1998	GAACcAAGTAAACCT	Adenomatous polyposis coli
2044	AGCTCcGCAATGCCAA	Adenomatous polyposis coli

2556	TCATCccttcctcGAGTAAGCAC	Adenomatous polyposis coli
2643	CTAATttatCAAATGGCAC	Adenomatous polyposis coli

TABLE X: SMALL INSERTIONS

Codon	Insertion	Phenotype
157	T	Adenomatous polyposis coli
170	AGAT	Adenomatous polyposis coli
172	T	Adenomatous polyposis coli
199	G	Adenomatous polyposis coli
243	AG	Adenomatous polyposis coli
266	T	Adenomatous polyposis coli
357	A	Adenomatous polyposis coli
405	C	Adenomatous polyposis coli
413	T	Adenomatous polyposis coli
416	A	Adenomatous polyposis coli
457	G	Adenomatous polyposis coli
473	A	Adenomatous polyposis coli
503	ATTC	Adenomatous polyposis coli
519	C	Adenomatous polyposis coli
528	A	Adenomatous polyposis coli
561	A	Adenomatous polyposis coli
608	A	Adenomatous polyposis coli
620	CT	Adenomatous polyposis coli
621	A	Adenomatous polyposis coli
623	TTAC	Adenomatous polyposis coli
627	A	Adenomatous polyposis coli
629	A	Adenomatous polyposis coli
636	GT	Adenomatous polyposis coli

639	A	Adenomatous polyposis coli
704	T	Adenomatous polyposis coli
740	ATGC	Adenomatous polyposis coli
764	T	Adenomatous polyposis coli
779	TT	Adenomatous polyposis coli
807	AT	Adenomatous polyposis coli
827	AT	Adenomatous polyposis coli
831	A	Adenomatous polyposis coli
841	CTTA	Adenomatous polyposis coli
865	CT	Adenomatous polyposis coli
865	AT	Adenomatous polyposis coli
900	TG	Adenomatous polyposis coli
921	G	Adenomatous polyposis coli
927	A	Adenomatous polyposis coli
935	A	Adenomatous polyposis coli
936	C	Adenomatous polyposis coli
975	A	Adenomatous polyposis coli
985	T	Adenomatous polyposis coli
997	A	Adenomatous polyposis coli
1010	TA	Adenomatous polyposis coli
1085	C	Adenomatous polyposis coli
1085	AT	Adenomatous polyposis coli
1095	A	Adenomatous polyposis coli
1100	GTTT	Adenomatous polyposis coli
1107	GGAG	Adenomatous polyposis coli
1120	G	Adenomatous polyposis coli
1166	A	Adenomatous polyposis coli
1179	T	Adenomatous polyposis coli
1187	A	Adenomatous polyposis coli
1211	T	Adenomatous polyposis coli
1256	A	Adenomatous polyposis coli

1265	T	Adenomatous polyposis coli
1267	GATA	Adenomatous polyposis coli
1268	T	Adenomatous polyposis coli
1301	A	Adenomatous polyposis coli
1301	C	Adenomatous polyposis coli
1323	A	Adenomatous polyposis coli
1342	T	Adenomatous polyposis coli
1382	T	Adenomatous polyposis coli
1458	GTAG	Adenomatous polyposis coli
1463	AG	Adenomatous polyposis coli
1488	T	Adenomatous polyposis coli
1531	A	Adenomatous polyposis coli
1533	T	Adenomatous polyposis coli
1554	A	Adenomatous polyposis coli
1555	A	Adenomatous polyposis coli
1556	T	Adenomatous polyposis coli
1563	GACCT	Adenomatous polyposis coli
1924	AA	Desmoid tumours

TABLE XI: SMALL INSERTIONS/DELETIONS

Location/ codon	**Deletion**	**Insertion**	**Phenotype**
538	**GAA**GAcTTACAGCAGG	gaa	Adenomatous polyposis coli
620	**CTTA**CttaCCGGAGCCAG	ct	Adenomatous polyposis coli
728	**AAT**ctcatGGCAAATAGG	Ttgcagctttaa	Adenomatous polyposis coli
971	**GAT**GgtTATGGTAAAA	taa	Adenomatous polyposis coli

TABLE XII: GROSS DELETIONS

2 kb including ex. 11	Adenomatous polyposis coli
3 kb I10E11-1.5 kb to I12E13-170 bp	Adenomatous polyposis coli
335 bp nt. 1409-1743 ex. 11-13	Adenomatous polyposis coli
6 kb incl. ex. 14	Adenomatous polyposis coli
817 bp I13E14-679 to I13E14+138	Adenomatous polyposis coli
ex. 11-15M	Adenomatous polyposis coli
ex. 11-3'UTR	Adenomatous polyposis coli
ex. 15A – ex. 15F	Adenomatous polyposis coli
ex. 4	Adenomatous polyposis coli
ex. 7, 8 and 9	Adenomatous polyposis coli
ex. 8 to beyond ex. 15F	Adenomatous polyposis coli
ex. 8 - ex. 15F	Adenomatous polyposis coli
ex. 9	Adenomatous polyposis coli
>10mb (del 5q22)	Adenomatous polyposis coli

TABLE XIII: GROSS INSERTIONS AND DUPLICATIONS

Description	**Phenotype**
Insertion of 14 bp nt. 3816	Adenomatous polyposis coli
Insertion of 22 bp nt. 4022	Adenomatous polyposis coli
Duplication of 43 bp cd. 1295	Adenomatous polyposis coli
Insertion of 337 bp of Alu I sequence cd. 1526	Desmoid tumours

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TABLE XIV: COMPLEX REARRANGEMENTS (INCLUDING INVERSIONS)

A-T nt. 4893 Q1625H, Del C nt. 4897 cd. 1627	Adenomatous polyposis coli
Del 1099 bp I13E14-728 to E14I14+156, ins 126 bp	Adenomatous polyposis coli

Del 1601 bp E14I14+27 to E14I14+1627, ins 180 bp	Adenomatous polyposis coli
Del 310 bp, ins. 15 bp nt. 4394, cd 1464	Adenomatous polyposis coli
Del A and T cd. 1395	Adenomatous polyposis coli
Del TC nt. 4145, Del TGT nt. 4148	Adenomatous polyposis coli
Del. T, nt. 983, Del. 70 bp, nt. 985	Adenomatous polyposis coli
Del. nt. 3892-3903, ins ATTT	Adenomatous polyposis coli

TABLE XV: DIAGNOSTIC APPLICATIONS

Cancer Type	**Marker**	**Application**	**Reference**
Breast	Her2/Neu Detection – polymorphism at codon 655 (GTC/valine to ATC/isoleucine [Val(655)Ile])	Using methods described herein, design second primer such that after PCR, and digestion with restriction enzyme, a 5' overhang containing DNA sequence for codon 655 of Her2/Neu is generated. Her2/Neu can be detected and quantified as a possible marker for breast cancer. Methods described herein can detect both mutant allele and normal allele, even when mutant allele is small fraction of total DNA. Herceptin therapy for breast cancer is based upon screening for Her2. The earlier the mutant allele can be detected, the faster therapy can be provided.	D. Xie *et al., J. Natl. Cancer Institute*,**92**, 412 (2000) K.S. Wilson *et al., Am. J. Pathol.*,**161**,1171 (2002) L. Newman, *Cancer Control*,**9**, 473 (2002)

Breast/Ovarian	Hypermethylation of BRCA1	Methods described herein can be used to differentiate between tumors resulting from inherited BRCA1 mutations and those from non-inherited abnormal methylation of the gene	M.Esteller *et al., New England Jnl Med.*, **344**, 539 (2001)
Bladder	Microsatellite analysis of free tumor DNA in Urine, Serum and Plasma	Methods described herein can be applied to microsatellite analysis and FGFR3 mutation analysis for detection of bladder cancer. Methods described herein provide a non-invasive method for detection of bladder cancer.	W.G. Bas *et al., Clinical Cancer Res.*,**9**,257 (2003) M. Utting *et al., Clincal Cancer Res.*, **8**,35 (2002) L. Mao, D.Sidransky *et al., Science*,**271**, 669 (1996)
Lung	Microsatellite analysis of DNA from sputum	Methods described herein can be used to detect mutations in sputum samples, and can markedly boost the accuracy of preclinical lung cancer screening	T.Liloglou *et al., Cancer Research*,**61**, 1624, (2001) M. Tockman *et al., Cancer Control*,7, 19 (2000)

			Field *et al.*, *Cancer Research*,**59**, 2690 (1999)
Cervical	Analysis of HPV genotype	Methods described herein can be used to detect HPV genotype from a cervical smear preparation.	N. Munoz *et al.*, *New England Jnl Med.*, **348**, 518 (2003)
Head and Neck	Tumor specific alterations in exfoliated oral mucosal cells (microsatellite markers)	Methods described herein can be used to detect any of 23 microsatellite markers, which are associated with Head and Neck Squamous Cell Carcinoma (HNSCC).	M. Spafford *et al. Clinical Cancer Research*,**17**, 607 (2001) A. El-Naggar *et al., J. Mol. Diag.*,**3**,164 (2001)
Colorectal	Screening for mutation in K-ras2 and APC genes.	Methods described herein can be used to detect K-ras 2 mutations, which can be used as a prognostic indicator for colorectal cancer. APC (see Example 5).	B. Ryan *et al. Gut*,**52**,101 (2003)
Prostate	GSTP1 Hypermethylation	Methods described herein can be used to detect GSTP1 hypermethylation in urine from patients with prostate cancer; this can be a more accurate indicator than PSA.	P. Cairns *et al. Clin. Can. Res.*,**7**,2727 (2001)

HIV

Antiretroviral resistance	Screening individuals for mutations in HIV virus – e.g. 154V mutation or CCR5 Δ 32 allele.	Methods described herein can be used for detection of mutations in the HIV virus. Treatment outcomes are improved in individuals receiving anti-retroviral therap based upon resistance screening.	J. Durant *et al. The Lancet*,353, 2195 (1999)

Cardiology

Congestive Heart Failure	Synergistic polymorphisms of beta1 and alpha2c adrenergic receptors	Methods described herein can be used to genotype these loci and may help identify people who are at a higher risk of heart failure.	K.Small *et al. New Eng. Jnl. Med.*, 347,1135 (2002)

EXAMPLE 8

Single nucleotide polymorphisms (SNPs) represent the most common form of sequence variation; three million common SNPs with a population frequency of over 5% have been estimated to be present in the human genome. A genetic map using these polymorphisms as a guide is being developed (http://research.marshfieldclinic.org/genetics/; internet address as of February 13, 2003).

The allele frequency varies from SNP to SNP; the allele frequency for one SNP may be 50:50, while the allele frequency for another SNP may be 90:10. The closer the allele frequency is to 50:50, the more likely any particular individual will be heterozygous at that SNP. The SNP consortium provides allele frequency information for some SNPs but not for others. www.snp.chsl.org. The allele frequency for a particular SNP provides valuable information as to the utility of that SNP for the non-invasive prenatal screening method described in Example 5. While all SNPs can be used, SNPs with allele frequencies closer to 50:50 are preferable.

Briefly, maternal blood contains fetal DNA. Maternal DNA can be distinguished from fetal DNA by examining SNPs wherein the mother is homozygous. For example, at SNP X, the maternal DNA may be homozygous for guanine. If template DNA obtained

from the plasma of a pregnant female is heterozygous, as demonstrated by the detection of signals corresponding to an adenine allele and an guanine allele, the adenine allele can be used as a beacon for the fetal DNA (see Example 5). The closer the allele frequency of a SNP is to 50:50, the more likely there will be allele differences at a particular SNP between the maternal DNA and the fetal DNA.

For example, if at SNP X the observed alleles are adenine and guanine, and the SNP has an allele frequency of 90(A):10(G), it is likely that both mother and father will be homozygous for adenine at that particular SNP. Thus, both the maternal DNA and the fetal DNA will be homozygous for adenine, and there is no distinct signal for the fetal DNA. However, if at SNP X the allele frequency is 50:50, and the mother is homozygous for adenine, the probability is higher that the paternal DNA will contain a guanine allele at SNP X.

Below, a method for determining the allele frequency for a SNP is provided. Seven SNPs located on chromosome 13 were analyzed. The method is applicable for any SNP including but not limited to the SNPs on human chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X and Y.

Preparation of Template DNA

To determine the allele frequency of a particular SNP, DNA was obtained from two hundred and fifty individuals after informed consent had been granted. From each individual, a 9 ml blood sample was collected into a sterile tube (Fischer Scientific, 9 ml EDTA Vacuette tubes, catalog number NC9897284). The tubes were spun at 1000 rpm for ten minutes. The supernatant (the plasma) of each sample was removed, and one milliliter of the remaining blood sample, which is commonly referred to as the "buffy-coat" was transferred to a new tube. One milliliter of 1X PBS was added to each sample.

Template DNA was isolated using the QIAmp DNA Blood Midi Kit supplied by QIAGEN (Catalog number 51183). The template DNA was isolated as per instructions included in the kit. From each individual, 0.76 μg of DNA was pooled together, and the pooled DNA was used in all subsequent reactions.

Design of Primers

SNP TSC0903430 was amplified using the following primer set:

First primer:

5' GTCTTGCATGTAGAATTCTAGGGACGCTGCTTTTCGTC 3'

Second primer:

5' CTCCTAGACATCGGGACTAGAATGTCCAC 3'

The first primer contained a recognition site for the restriction enzyme EcoRI, and was designed to anneal eighty-two bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC0337961 was amplified using the following primer set:

First primer:

5' ACACAAGGCAGAGAATTCCAGTCCTGAGGGTGGGGGCC 3'

Second primer:

5' CCGTGTTTTAACGGGACAAGCTGTTCTTC 3'

The first primer contained a recognition site for the restriction enzyme EcoRI, and was designed to anneal ninety-two bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC0786441 was amplified using the following primer set:

First primer:

5' GTAGCGGAGGTTGAATTCTATATGTTGTCTTGGACATT 3'

Second primer:

5' CATCAGTAGAGTGGGACGAAAGTTCTGGC 3'

The first primer contained a recognition site for the restriction enzyme EcoRI, and was designed to anneal one hundred and four bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC1168303 was amplified using the following primer set:

First primer:

5' ATCCACGCCGCAGAATTCGTATTCATGGGCATGTCAAA 3'

Second primer:

5' CTTGGGACTATTGGGACCAGTGTTCAATC 3'

The first primer contained a recognition site for the restriction enzyme EcoRI, and was designed to anneal sixty-four bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC0056188 was amplified using the following primer set:

First primer:

5' CCAGAAAGCCGTGAATTCGTTAAGCCAACCTGACTCCA 3'

Second primer:

5' TCGGGGTTAGTCGGGACATCCAGCAGCCC 3'

The first primer contained a recognition site for the restriction enzyme EcoRI, and was designed to anneal eighty-two bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC0466177 was amplified using the following primer set:

First primer:

5' CGAAGGTAATGTGAATTCCAAAACTTAGTGCCACAATT 3'

Second primer:

5' ATACCGCCCAACGGGACAGATCCATTGAC 3'

The first primer contained a recognition site for the restriction enzyme EcoRI, and was designed to anneal ninety-two bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC0197424 was amplified using the following primer set:

First primer:

5' AGAAACCTGTAAGAATTCGATTCCAAATTGTTTTTTGG 3'

Second primer:

5' CGATCATAGGGGGGGACAGGAGAGAGCAC 3'

The first primer contained a recognition site for the restriction enzyme EcoRI, and was designed to anneal one hundred and four bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

The first primer was designed to anneal at various distances from the locus of interest. The skilled artisan understands that the annealing location of the first primer can

be any distance from the locus of interest including but not limited to 5-10, 11-15, 16-20, 21-25, 26-30, 31-35, 36-40, 41-45, 46-50, 51-55, 56-60, 61-65, 66-70, 71-75, 76-80, 81-85, 86-90, 91-95, 96-100, 101-105, 106-110, 111-115, 116-120, 121-125, 126-130, 131-140, 141-160, 161-180, 181-200, 201-220, 221-240, 241-260, 261-280, 281-300, 301-350, 351-400, 401-450, 451-500, 501-1000, 1001-2000, 2001-3000, or greater than 3000.

All loci of interest were amplified from the template genomic DNA using the polymerase chain reaction (PCR, U.S. Patent Nos. 4,683,195 and 4,683,202, incorporated herein by reference). In this example, the loci of interest were amplified in separate reaction tubes but they can also be amplified together in a single PCR reaction. For increased specificity, a "hot-start" PCR was used. PCR reactions were performed using the HotStarTaq Master Mix Kit supplied by QIAGEN (catalog number 203443). The amount of template DNA and primer per reaction can be optimized for each locus of interest. In this example, 40 ng of template human genomic DNA (a mixture of template DNA from 245 individuals) and 5 μM of each primer were used. Forty cycles of PCR were performed. The following PCR conditions were used:

(1) 95°C for 15 minutes and 15 seconds;
(2) 37°C for 30 seconds;
(3) 95°C for 30 seconds;
(4) 57°C for 30 seconds;
(5) 95°C for 30 seconds;
(6) 64°C for 30 seconds;
(7) 95°C for 30 seconds;
(8) Repeat steps 6 and 7 thirty nine (39) times;
(9) 72°C for 5 minutes.

In the first cycle of PCR, the annealing temperature was about the melting temperature of the 3′ annealing region of the second primers, which was 37°C. The annealing temperature in the second cycle of PCR was about the melting temperature of the 3' region, which anneals to the template DNA, of the first primer, which was 57°C. The annealing temperature in the third cycle of PCR was about the melting temperature of the entire sequence of the second primer, which was 64°C. The annealing temperature for the remaining cycles was 64°C. Escalating the annealing temperature from TM1 to TM2 to TM3 in the first three cycles of PCR greatly improves specificity. These

annealing temperatures are representative, and the skilled artisan will understand the annealing temperatures for each cycle are dependent on the specific primers used.

The temperatures and times for denaturing, annealing, and extension, can be optimized by trying various settings and using the parameters that yield the best results.

Purification of Fragment of Interest

The PCR products were separated from the unused PCR reagents. After the PCR reaction, 1/2 of the reaction volume for SNP TSC0903430, SNP TSC0337961, and SNP TSC0786441 were mixed together in a single reaction tube. One-half the reaction volumes for SNPs TSC1168303, TSC0056188, TSC0466177, and TSC0197424 were pooled together in a single reaction tube. The un-used primers, and nucleotides were removed from the reaction by using Qiagen MinElute PCR purification kits (Qiagen, Catalog Number 28004). The reactions were performed following the manufacturer's instructions supplied with the columns.

Restriction Enzyme Digestion of Isolated Fragments

The purified PCR products were digested with the restriction enzyme BsmF I, which binds to the recognition site incorporated into the PCR products from the second primer. The digests were performed in eppendorf tubes following the instructions supplied with the restriction enzyme.

Incorporation of Labeled Nucleotide

The restriction enzyme digest with BsmF I yielded a DNA fragment with a 5' overhang, which contained the SNP site or locus of interest and a 3' recessed end. The 5' overhang functioned as a template allowing incorporation of a nucleotide or nucleotides in the presence of a DNA polymerase.

As discussed in detail in Example 6, the sequence of both alleles of a SNP can be determined with one labeled nucleotide in the presence of the other unlabeled nucleotides. The following components were added to each fill in reaction: 1 µl of fluorescently labeled ddGTP, 0.5 µl of unlabeled ddNTPs (40 µM), which contained all nucleotides except guanine, 2 µl of 10X sequenase buffer, 0.25 µl of Sequenase, and water as needed for a 20µl reaction. The fill in reaction was performed at 40°C for 10 min. Sequenase was the DNA polymerase used in this example. However, any DNA

polymerase can be used for a fill-in reaction including but not limited to E. coli DNA polymerase, Klenow fragment of E. coli DNA polymerase I, T7 DNA polymerase, T4 DNA polymerase, Taq polymerase, Pfu DNA polymerase, Vent DNA polymerase, polymerase from bacteriophage 29, and REDTaq™ Genomic DNA polymerase. Non-fluorescently labeled ddNTP was purchased from Fermentas Inc. (Hanover, MD). All other labeling reagents were obtained from Amersham (Thermo Sequenase Dye Terminator Cycle Sequencing Core Kit, US 79565).

Detection of the Locus of Interest

The sample was loaded into a lane of a 36 cm 5% acrylamide (urea) gel (BioWhittaker Molecular Applications, Long Ranger Run Gel Packs, catalog number 50691). The sample was electrophoresed into the gel at 3000 volts for 3 min. The gel was run for 3 hours on a sequencing apparatus (Hoefer SQ3 Sequencer). The gel was removed from the apparatus and scanned on the Typhoon 9400 Variable Mode Imager. The incorporated labeled nucleotide was detected by fluorescence.

Below, a schematic of the 5' overhang for SNP TSC0056188 is reproduced (where R indicates the variable site). The entire sequence is not shown, only a portion of the overhang.

5'CCA				
3'GGT	R	T	C	C
Overhang position	1	2	3	4

As discussed in detail in Example 6, one nucleotide labeled with one chemical moiety can be used to determine the sequence of the alleles of a locus of interest. The observed nucleotides for TSC0056188 on the 5' sense strand (here depicted as the top strand) are adenine and guanine. The third position in the overhang on the antisense strand is cytosine, which is complementary to guanine. As the variable site can be adenine or guanine, fluorescently labeled ddGTP in the presence of unlabeled dCTP, dTTP, and dATP was used to determine the sequence of both alleles. The fill-in reactions for an individual homozygous for guanine, homozygous for adenine or heterozygous are diagrammed below.

Homozygous adenine:

	5'CCA	**A**	A	**G***	
	3'GGT	T	T	C	C
Overhang position		1	2	3	4

Homozygous guanine:

	5'CCA	**G***			
	3'GGT	C	T	C	C
Overhang position		1	2	3	4

Heterozygous:

Allele 1	5'CCA	**G***				
	3'GGT	C	T	C	C
Overhang position		1	2	3	4
Allele 2	5'CCA	**A**	A	**G***	
	3'GGT	T	T	C	C
Overhang position		1	2	3	4

As seen in FIG. 14, two bands were detected for SNP TSC0056188. The lower band corresponded to DNA molecules filled in with ddGTP at position one complementary to the overhang, which is representative of the guanine allele. The higher band, separated by a single base from the lower band, corresponded to DNA molecules filled in with ddGTP at position 3 complementary to the overhang. This band represented the adenine allele. The intensity of each band was strong, indicating that each allele was well represented in the population. SNP TSC0056188 is representative of a SNP with high allele frequency.

Below, a schematic of the 5' overhang generated after digestion with BsmF I for SNP TSC0337961 is reproduced (where R indicates the variable site). The entire sequence is not shown, only a portion of the overhang.

	5' GCCA				
	3' CGGT	R	G	C	T
Overhang position		1	2	3	4

The observed nucleotides for SNP TSC0337961 on the 5' sense strand (here depicted as the top strand) are adenine and guanine. The third position in the overhang on the antisense strand was cytosine, which is complementary to guanine. As the variable site can be adenine or guanine, fluorescently labeled ddGTP in the presence of unlabeled dCTP, dTTP, and dATP was used to determine the sequence of both alleles. The fill-in reactions for an individual homozygous for guanine, homozygous for adenine or heterozygous are diagrammed below.

Homozygous for guanine:

	5' GCCA	**G***			
	3' CGGT	C	G	C	T
Overhang position		1	2	3	4

Homozygous for adenine:

	5' GCCA	**A**	C	**G***	
	3' CGGT	T	G	C	T
Overhang position		1	2	3	4

Heterozygous

Allele 1	5' GCCA	**G***			
	3' CGGT	C	G	C	T
Overhang position		1	2	3	4
Allele 2	5' GCCA	**A**	C	**G***	
	3' CGGT	T	G	C	T

Overhang position 1 2 3 4

As seen in FIG. 14, one band migrating at the position of the expected lower molecular weight band was observed. This band represented the DNA molecules filled in with ddGTP at position one complementary to the overhang, which represents the guanine allele. No band corresponding to the DNA molecules filled in with ddGTP at position 3 complementary to the overhang was detected. SNP TSC0337961 is representative of a SNP that is not highly variable within the population.

Of the seven SNPs analyzed, four of the SNPs (TSC1168303, TSC0056188, TSC0466177, and TSC0197424 had high allele frequencies. Two bands of high intensity were seen for each of the four SNPs, indicating that both alleles were well represented in the population.

However, it is not necessary that the SNPs have allele frequencies of 50:50 to be useful. All SNPs provide useful information. The methods described herein provide a rapid technique for determining the allele frequency of a SNP, or any variable site including but not limited to point mutations. Allele frequencies of 50:50, 51:49, 52:48, 53:47, 54:46, 55:45, 56:46, 57:43, 58:42, 59:41, 60:40, 61:39, 62:38, 63:37, 64:36, 65:35, 66:34, 67:33, 68:32, 69:31, 70:30, 71:29, 72:28, 73:27, 74:26, 75:25, 76:24, 77:23, 78:22, 79:21, 80:20, 81:19, 82:18, 83:17, 84:16, 85:15, 86:14, 87:13, 88:12, 89:11, 90:10, 91:9, 92:8, 93:7, 94:6, 95:5, 96:4, 97:3, 98:2, 99:1 and 100:0 can be useful.

Two bands were seen for SNP TSC0903430. One band, the lower molecular weight band represented the DNA molecules filled in with labeled ddGTP. A band of weaker intensity was seen for the molecules filled in with labeled ddGTP at position 3 complementary to the overhang, which represented the cytosine allele. SNP TSC0903430 represents a SNP with low allele frequency variation. In the population, the majority of individuals carry the guanine allele, but the cytosine allele is still present.

One band of high intensity was seen for SNP TSC0337961 and SNP TSC0786441. The band detected for both SNP TSC0337961 and SNP TSC0786441 corresponded to the DNA molecules filled in with ddGTP at position 1 complementary to the overhang. No signal was detected from DNA molecules that would have been filled in at position 3 complementary to the overhang, which would have represented the second allele. SNP TSC0337961 and SNP TSC0786441 represent SNPs with little variability in the population.

As demonstrated in FIG 14., the first primer used to amplify each locus of interest can be designed to anneal at various distances from the locus of interest. This allows multiple SNPs to be analyzed in the same reaction. By designing the first primer to anneal at specified distances from the loci of interest, any number of loci of interest can be analyzed in a single reaction including but not limited to 1-10, 11-20, 21-30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90, 91-100, 101-110, 111-120, 121-130, 131-140, 141-150, 151-160, 161-170, 171-180, 181-190, 191-200, 201-300, 301-400, 401-500, and greater than 500.

As discussed in Example 6, some type IIs restriction enzymes display alternate cutting patterns. For example, the type IIS restriction enzyme BsmF I typically cuts 10/14 from its binding site; however, the enzyme also can cut 11/15 from the binding site. To eliminate the effect of the alternate cut, the labeled nucleotide used for the fill-in reaction should be chosen such that it is not complementary to position 0 of the overhang generated by the 11/15 cut (discussed in detail in Example 6). For instance, if you label with ddGTP, the nucleotide preceding the variable site on the strand that is filled in should not be a guanine.

The 11/15 overhang generated by BsmF I for SNP TSC0056188 is depicted below, with the variable site in bold-typeface:

11/15 Overhang for TSC0056188

Allele 1	5'CC				
	3'GG	T	**C**	T	C
Overhang position		0	1	2	3
Allele 2	5'CC				
	3'GG	T	**T**	T	C
Overhang position		0	1	2	3

After the fill-in reaction with labeled ddGTP, unlabeled dATP, dTTP, and dCTP, the following molecules were generated:

11/15 Allele 1	5'CC	A	**G***

		3'GG	T	C	T	C
		Overhang position	0	1	2	3
	11/15 Allele 2	5'CC	A	A	A	G*
		3'GG	T	T	T	C
		Overhang position	0	1	2	3

Two signals were seen; one band corresponded to molecules filled in with ddGTP at position one of the overhang, and the other band corresponded to the molecules filled in with ddGTP at position 3 complementary to the overhang. These are the same DNA molecules generated after the fill-in reaction of the 10/14 overhang. Thus, the two bands can be compared without any ambiguity from the alternate cut. This method of labeling with a single nucleotide eliminates any errors generated from the alternate cutting properties of the enzymes.

The methods described herein is applicable to determining the allele frequency of any SNP including but not limited to SNPs on human chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X and Y.

EXAMPLE 9

Heterozygous SNPs, by definition, differ by one nucleotide. At a heterozygous SNP, allele 1 and allele 2 may be present at a ratio of 1:1. However, it is possible that DNA polymerases can incorporate one nucleotide at a faster rate than other nucleotides, and thus the observed ratio of a heterozygous SNP may differ from the theoretically expected 1:1 ratio.

Below, methods are described that allow efficient and accurate quantitation for the expected ratio of allele 1 to allele 2 at a heterozygous SNP.

Preparation of Template DNA

Template DNA was obtained from twenty-four individuals after informed consent had been granted. From each individual, a 9 ml blood sample was collected into a sterile tube (Fischer Scientific, 9 ml EDTA Vacuette tubes, catalog number

NC9897284). The tubes were spun at 1000 rpm for ten minutes without brake. The supernatant (the plasma) of each sample was removed, and one milliliter of the remaining blood sample, which is commonly referred to as the "buffy-coat" was transferred to a new tube. One milliliter of 1X PBS was added to each sample.

Template DNA was isolated using the QIAmp DNA Blood Midi Kit supplied by QIAGEN (Catalog number 51183). The template DNA was isolated as per instructions included in the kit.

Design of Primers

SNP TSC0607185 was amplified using the following primer set:

First primer:

5' ACTTGATTCCGTGAATTCGTTATCAATAAATCTTACAT 3'

Second primer:

5' CAAGTTGGATCCGGGACCCAGGGCTAACC 3'

SNP TSC1130902 was amplified using the following primer set:

First primer:

5' TCTAACCATTGCGAATTCAGGGCAAGGGGGGTGAGATC 3'

Second primer:

5' TGACTTGGATCCGGGACAACGACTCATCC 3'

The first primer contained a biotin tag at the 5' end and a recognition site for the restriction enzyme EcoRI. The second primer contained the recognition site for the

restriction enzyme BsmF I. The first primer was designed to anneal at various distances from the locus of interest.

The first primer for SNP TSC0607185 was designed to anneal ninety bases from the locus of interest. The first primer for SNP TSC1130902 was designed to anneal sixty bases from the locus of interest.

All loci of interest were amplified from the template genomic DNA using the polymerase chain reaction (PCR, U.S. Patent Nos. 4,683,195 and 4,683,202, incorporated herein by reference). In this example, the loci of interest were amplified in separate reaction tubes but they could also be amplified together in a single PCR reaction. For increased specificity, a "hot-start" PCR was used. PCR reactions were performed using the HotStarTaq Master Mix Kit supplied by QIAGEN (catalog number 203443). The amount of template DNA and primer per reaction can be optimized for each locus of interest but in this example, 40 ng of template human genomic DNA and 5 μM of each primer were used. Forty cycles of PCR were performed. The following PCR conditions were used:

(1) 95°C for 15 minutes and 15 seconds;
(2) 37°C for 30 seconds;
(3) 95°C for 30 seconds;
(4) 57°C for 30 seconds;
(5) 95°C for 30 seconds;
(6) 64°C for 30 seconds;
(7) 95°C for 30 seconds;
(8) Repeat steps 6 and 7 thirty nine (39) times;
(9) 72°C for 5 minutes.

In the first cycle of PCR, the annealing temperature was about the melting temperature of the 3′ annealing region of the second primers, which was 37°C. The annealing temperature in the second cycle of PCR was about the melting temperature of the 3' region, which anneals to the template DNA, of the first primer, which was 57°C. The annealing temperature in the third cycle of PCR was about the melting temperature of the entire sequence of the second primer, which was 64°C. The annealing temperature for the remaining cycles was 64°C. Escalating the annealing temperature from TM1 to TM2 to TM3 in the first three cycles of PCR greatly improves specificity. These

annealing temperatures are representative, and the skilled artisan will understand the annealing temperatures for each cycle are dependent on the specific primers used.

The temperatures and times for denaturing, annealing, and extension, can be optimized by trying various settings and using the parameters that yield the best results.

Purification of Fragment of Interest

The PCR products were separated from the genomic template DNA. One half of the PCR reaction was transferred to a well of a Streptawell, transparent, High-Bind plate from Roche Diagnostics GmbH (catalog number 1 645 692, as listed in Roche Molecular Biochemicals, 2001 Biochemicals Catalog). The first primers contained a 5' biotin tag so the PCR products bound to the Streptavidin coated wells while the genomic template DNA did not. The streptavidin binding reaction was performed using a Thermomixer (Eppendorf) at 1000 rpm for 20 min. at 37°C. Each well was aspirated to remove unbound material, and washed three times with 1X PBS, with gentle mixing (Kandpal et al., Nucl. Acids Res. 18:1789-1795 (1990); Kaneoka et al., Biotechniques 10:30-34 (1991); Green et al., Nucl. Acids Res. 18:6163-6164 (1990)).

Restriction Enzyme Digestion of Isolated Fragments

The purified PCR products were digested with the restriction enzyme BsmF I, which binds to the recognition site incorporated into the PCR products from the second primer. The digests were performed in the Streptawells following the instructions supplied with the restriction enzyme. After digestion, the wells were washed three times with PBS to remove the cleaved fragments.

Incorporation of Labeled Nucleotide

The restriction enzyme digest with BsmF I yielded a DNA fragment with a 5' overhang, which contained the SNP site or locus of interest and a 3' recessed end. The 5' overhang functioned as a template allowing incorporation of a nucleotide or nucleotides in the presence of a DNA polymerase.

As discussed in detail in Example 6, the sequence of both alleles of a SNP can be determined by using one labeled nucleotide in the presence of the other unlabeled nucleotides. The following components were added to each fill in reaction: 1 µl of fluorescently labeled ddGTP, 0.5 µl of unlabeled ddNTPs (40 µM), which contained all

nucleotides except guanine, 2 µl of 10X sequenase buffer, 0.25 µl of Sequenase, and water as needed for a 20µl reaction. The fill in reaction was performed at 40°C for 10 min. Non-fluorescently labeled ddNTP was purchased from Fermentas Inc. (Hanover, MD). All other labeling reagents were obtained from Amersham (Thermo Sequenase Dye Terminator Cycle Sequencing Core Kit, US 79565).

After labeling, each Streptawell was rinsed with 1X PBS (100 µl) three times. The "filled in" DNA fragments were then released from the Streptawells by digestion with the restriction enzyme EcoRI, according to the manufacturer's instructions that were supplied with the enzyme. Digestion was performed for 1 hour at 37 °C with shaking at 120 rpm.

Detection of the Locus of Interest

The samples were loaded into a lane of a 36 cm 5% acrylamide (urea) gel (BioWhittaker Molecular Applications, Long Ranger Run Gel Packs, catalog number 50691). The samples were electrophoresed into the gel at 3000 volts for 3 min. The gel was run for 3 hours on a sequencing apparatus (Hoefer SQ3 Sequencer). The gel was removed from the apparatus and scanned on the Typhoon 9400 Variable Mode Imager. The incorporated labeled nucleotide was detected by fluorescence. A box was drawn around each band and the intensity of the band was calculated using the Typhoon 9400 Variable Mode Imager software.

Below, a schematic of the 5' overhang for SNP TSC0607185 is shown. The entire DNA sequence is not reproduced, only the portion to demonstrate the overhang (where R indicates the variable site).

	C	C	T	R	TGTC 3'
					ACAG 5'
	4	3	2	1	Overhang position

The observed nucleotides at the variable site for TSC0607185 on the 5' sense strand (here depicted as the top strand) are cytosine and thymidine (depicted here as R). In this case, the second primer anneals from the locus of interest, which allows the fill-in reaction to occur on the anti-sense strand (depicted here as the bottom strand). The antisense strand will be filled in with guanine or adenine.

The second position in the 5' overhang is thymidine, which is complementary to adenine, and the third position in the overhang corresponds to cytosine, which is complementary to guanine. Fluorescently labeled ddGTP in the presence of unlabeled dCTP, dTTP, and dATP was used to determine the sequence of both alleles. After the fill-in reaction, the following DNA molecules were generated:

C	C	T	C	TGTC 3'	Allele 1
			G*	ACAG 5'	
4	3	2	1	Overhang position	

C	C	T	T	TGTC 3'	Allele 1
	G*	A	A	ACAG 5'	
4	3	2	1	Overhang position	

The overhang generated by BsmF I cutting at 11/15 from the recognition site at TSC0607185 is depicted below:

C	T	R	T	GTC 3' 11/15
				CAG 5'
3	2	1	0	Overhang position

As labeled ddGTP is used for the fill-in reaction, no new signal will be generated from the molecules cut 11/15 from the recognition site. Position 0 complementary to the overhang was filled in with unlabeled dATP. Only signals generated from molecules filled in with labeled ddGTP at position 1 complementary to the overhang or molecules filled in with labeled ddGTP at position 3 complementary to the overhang were seen.

Five of the twenty-four individuals were heterozygous for SNP TSC0607185. As shown in FIG. 15, two bands were detected. The lower molecular weight band corresponded to DNA molecules filled in with ddGTP at position 1 complementary to the overhang. The higher molecular weight band corresponded to DNA molecules filled in with ddGTP at position 3 complementary to the overhang.

The ratio of the two alleles was calculated for each of the five heterozygous samples (see Table XVI). The average ratio of allele 2 to allele 1 was 1.000 with a

standard deviation of 0.044. Thus, the allele ratio at SNP TSC0607185 was highly consistent. The experimentally calculated allele ratio for a particular SNP is hereinafter referred to as the "p" value of the SNP. Analysis of SNP TSC0607185 consistently will provide an allele ratio of 1:1, provided that the number of genomes analyzed is of sufficient quantity that no error is generated from statistical sampling.

If the sample contained a low number of genomes, it is statistically possible that the primers will anneal to one chromosome over another chromosome. For example, if the sample contains 40 genomes, which corresponds to a total of 40 chromosomes of allele 1 and 40 chromosomes of allele 2, the primers may anneal to 40 chromosomes of allele 1 but only 35 chromosome of allele 2. This would cause allele 1 to be amplified preferentially to allele 2, which would alter the ratio of allele 1 to allele 2. This problem is eliminated by having a sufficient number of genomes in the sample.

SNP TSC0607185 represents a SNP where the difference in the nucleotide at the variable site does not affect the PCR reaction, or digestion with the restriction enzyme or the fill-in reaction. The use of one nucleotide labeled with one fluorescent dye assures that the bands for one allele can be accurately compared to the bands for the second allele. There is no added complication of having to compare between two different lanes, or having to correct for the quantum coefficients of the dyes. Additionally, any effect from the alternate cutting properties of the type IIS restriction enzymes has been removed.

TABLE XVI. Ratio of allele 2 to allele 1 at SNPs TSC0607185 and TSC1130902.

	SNP TSC0607185			SNP TSC1130902		
Sample	Allele 1	Allele 2	Allele2/Allele 1	Allele 1	Allele 2	Allele2/Allele 1
1	2382	2313	0.971033	5877	4433	0.754296
2	1581	1533	0.969639	3652	2695	0.737952
3	1795	1879	1.046797	5416	3964	0.730059
4	1921	1855	0.965643	3493	2663	0.762382
5	1618	1701	1.051298	3894	2808	0.721109
Average			1.000882			0.74116

STD			0.044042			0.017018

Below, a schematic of the 5' overhang for SNP TSC1130902 is shown. The entire DNA sequence is not reproduced, only the portion to demonstrate the overhang (where R indicates the variable site).

	5' TTCAT				
	3' AAGTA	R	T	C	C
Overhang position		1	2	3	4

The observed nucleotides for TSC1130902 on the 5' sense strand (here depicted as the top strand) are adenine and guanine. The second position in the overhang corresponds to a thymidine, and the third position in the overhang corresponds to cytosine, which is complementary to guanine. Fluorescently labeled ddGTP in the presence of unlabeled dCTP, dTTP, and dATP was used to determine the sequence of both alleles. After the fill-in reaction, the following DNA molecules were generated:

Allele 1	5' TTCAT	G*			
	3' AAGTA	C	T	C	C
Overhang position		1	2	3	4

Allele 2	5' TTCAT	A	A	G*	
	3' AAGTA	T	T	C	C
Overhang position		1	2	3	4

As shown in FIG. 15, two bands were detected. The lower molecular weight band corresponded to DNA molecules filled in with labeled ddGTP at position 1 complementary to the overhang (the G allele). The higher molecular weight band, separated by a single base from the lower band, corresponded to DNA molecules filled in with ddGTP at position 3 complementary to the overhang (the A allele).

Five of the twenty-four individuals were heterozygous for SNP TSC1130902. As seen in FIG. 15, the band corresponding to allele 1 was more intense than the band corresponding to allele 2. This was seen for each of the five individuals. The actual

intensity of the band corresponding to allele 1 varied from individual to individual but it was always more intense than the band corresponding to allele 2. For the five individuals, the average ratio of allele 2 to allele 1 was 0.74116, with a standard deviation of 0.017018.

Template DNA was prepared from five different individuals. Separate PCR reactions, separate restriction enzyme digestions, and separate fill-in reactions were performed. However, for each template DNA, the ratio of allele 2 to allele 1 was about 0.75. The "p" value for this SNP was highly consistent.

For example, for SNP TSC1130902, the "p" value was 0.75. Any deviation from this value, provided the sample contains an adequate number of genomes to remove statistical sampling errors, will indicate that there is an abnormal copy number of chromosome 13. If there is an additional copy of allele 2, the "p" value will be higher than the expected 0.75. However, if there is an addition copy of allele 1, the "p" value will be lower than the expected 0.75. With the "p" value quantitated for a particular SNP, that SNP can be used to determine the presence or absence of a chromosomal abnormality. An accurate "p" value measured for a single SNP will be sufficient to detect the presence of a chromosomal abnormality.

There are several possible explanations for why the ratio of one allele to the other allele at some SNPs varies from the theoretically expected ratio of 1:1. First, it is possible that the DNA polymerase incorporates one nucleotide faster than the other nucleotide. As the alleles are being amplified by PCR, even a slight preference for one nucleotide over the other may cause variation from the expected 1:1 ratio. This potential preference for one nucleotide over the other is not seen during the fill-in reaction because a single nucleotide labeled with one dye is used.

It is also possible that the variable nucleotide at the SNP site influences the rate of denaturation of the two alleles. If allele 1 contains a guanine and allele 2 contains an adenine, the difference between the strength of the bonds for these nucleotides may affect the rate at which the DNA strands separate. Again, it is important to mention that the alleles are being amplified by PCR so very subtle differences can make a large impact on the final result. It is also possible that the variable nucleotide at the SNP site influences the rate at which the two strands anneal after separation.

Alternatively, it is possible that the type IIS restriction enzyme cuts one allele preferentially to the other allele. As discussed in detail above, type IIS restriction

enzymes cut at a distance from the recognition site. It is possible that the variable nucleotide at the SNP site influences the efficiency of the restriction enzyme digestion. It is possible that at some SNPs the restriction enzyme cuts one allele with an efficiency of 100%, while it cuts the other allele with an efficiency of 90%.

However, the fact that the ratio of allele 1 to allele 2 deviates from the theoretically expected ratio of 1:1, does not influence or reduce the utility of that SNP. As demonstrated above, the "p" value for each SNP is consistent among different individuals.

The "p" value for any SNP can be calculated by analyzing the template DNA of any number of heterozygous individuals including but not limited to 1-10, 11-20, 21-30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90, 91-100, 101-110, 111-120, 121-130, 131-140, 141-150, 151-160, 161-170, 171-180, 181-190, 191-200, 201-210, 211-220, 221-230, 231-240, 241-250, 251-260, 261-270, 271-280, 281-290, 291-300, and greater than 300.

The methods described herein allow the "p" value for any SNP to be determined. It is possible that some SNPs will behave more consistently than other SNPs. In the human genome, there are over 3 million SNPs; it is not possible to speculate on how each SNP will behave. The "p" value for each SNP will have to be experimentally determined. The methods described herein allow identification of SNPs that have highly consistent, and reproducible "p" values.

EXAMPLE 10

As discussed in Example 9, the ratio of one allele to the other allele at a particular SNP may vary from the theoretically expected ratio of 50:50. These SNPs can be used to detect the presence of additional chromosomes provided that the ratio of one allele to the other allele remains linear in individuals with chromosomal disorders. For example, at SNP X if the percentage of allele 1 to allele 2 is 75:25, the expected percentage of allele 1 to allele 2 for an individual with Down's syndrome must be properly adjusted to reflect the variation from the expected percentage at this SNP.

The percentage of allele 1 to allele 2 for SNP TSC0108992 on chromosome 21 was calculated using template DNA from four normal individuals and template DNA from an individual with Down's syndrome. As demonstrated below, the percentage of

one allele to the other allele was consistent and remained linear in an individual with Down's syndrome.

Preparation of Template DNA

DNA was obtained from four individuals with a normal genetic karyotype and an individual identified as having an extra copy of chromosome 21 (Down's syndrome). Informed consent was obtained from all individuals. Informed consent also was obtained from the parents of the individual with Down's syndrome.

From each individual, a 9 ml blood sample was collected into a sterile tube (Fischer Scientific, 9 ml EDTA Vacuette tubes, catalog number NC9897284). Template DNA was isolated using the QIAmp DNA Blood Midi Kit supplied by QIAGEN (Catalog number 51183). The template DNA was isolated as per instructions included in the kit.

Design of Primers

SNP TSC0108992 was amplified using the following primer set:

First primer:

5' CTACTGAGGGCTCGTAGATCCCAATTCCTTCCCAAGCT 3'

Second primer:

5' AATCCTGCTTTAGGGACCATGCTGGTGGA 3'

The first primer contained a biotin tag at the 5' end and a recognition site for the restriction enzyme EcoRI. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC0108992 was amplified from the template genomic DNA using the polymerase chain reaction (PCR, U.S. Patent Nos. 4,683,195 and 4,683,202, incorporated herein by reference). For increased specificity, a "hot-start" PCR was used. PCR reactions were performed using the HotStarTaq Master Mix Kit supplied by QIAGEN

(catalog number 203443). The amount of template DNA and primer per reaction can be optimized for each locus of interest. In this example, 50 ng of template human genomic DNA and 5 μM of each primer were used. Thirty-eight cycles of PCR were performed. The following PCR conditions were used:

(1) 95°C for 15 minutes and 15 seconds;
(2) 37°C for 30 seconds;
(3) 95°C for 30 seconds;
(4) 57°C for 30 seconds;
(5) 95°C for 30 seconds;
(6) 64°C for 30 seconds;
(7) 95°C for 30 seconds;
(8) Repeat steps 6 and 7 thirty-seven (37) times;
(9) 72°C for 5 minutes.

In the first cycle of PCR, the annealing temperature was about the melting temperature of the 3′ annealing region of the second primers, which was 37°C. The annealing temperature in the second cycle of PCR was about the melting temperature of the 3' region, which anneals to the template DNA, of the first primer, which was 57°C. The annealing temperature in the third cycle of PCR was about the melting temperature of the entire sequence of the second primer, which was 64°C. The annealing temperature for the remaining cycles was 64°C. Escalating the annealing temperature from TM1 to TM2 to TM3 in the first three cycles of PCR greatly improves specificity. These annealing temperatures are representative, and the skilled artisan will understand the annealing temperatures for each cycle are dependent on the specific primers used.

The temperatures and times for denaturing, annealing, and extension, can be optimized by trying various settings and using the parameters that yield the best results.

Purification of Fragment of Interest

The PCR products were separated from the genomic template DNA. Each PCR reaction was split into two samples and transferred to two separate wells of a Streptawell, transparent, High-Bind plate from Roche Diagnostics GmbH (catalog number 1 645 692, as listed in Roche Molecular Biochemicals, 2001 Biochemicals Catalog). For each PCR reaction, there were two replicates; each in a separate well of a microtiter plate. The first primer contained a 5' biotin tag so the PCR products bound to the Streptavidin coated

wells while the genomic template DNA did not. The streptavidin binding reaction was performed using a Thermomixer (Eppendorf) at 1000 rpm for 20 min. at 37°C. Each well was aspirated to remove unbound material, and washed three times with 1X PBS, with gentle mixing (Kandpal et al., Nucl. Acids Res. 18:1789-1795 (1990); Kaneoka et al., Biotechniques 10:30-34 (1991); Green et al., Nucl. Acids Res. 18:6163-6164 (1990)).

Restriction Enzyme Digestion of Isolated Fragments

The purified PCR products were digested with the restriction enzyme BsmF I, which binds to the recognition site incorporated into the PCR products from the second primer. The digests were performed in the Streptawells following the instructions supplied with the restriction enzyme. After digestion, the wells were washed three times with 1X PBS to remove the cleaved fragments.

Incorporation of Labeled Nucleotide

The restriction enzyme digest with BsmF I yielded a DNA fragment with a 5' overhang, which contained the SNP site or locus of interest and a 3' recessed end. The 5' overhang functioned as a template allowing incorporation of a nucleotide or nucleotides in the presence of a DNA polymerase.

As discussed in detail in Example 6, the sequence of both alleles of a SNP can be determined with one labeled nucleotide in the presence of the other unlabeled nucleotides. The following components were added to each fill in reaction: 1 µl of fluorescently labeled ddTTP, 0.5 µl of unlabeled ddNTPs (40 µM), which contained all nucleotides except thymidine, 2 µl of 10X sequenase buffer, 0.25 µl of Sequenase, and water as needed for a 20µl reaction. The fill in reaction was performed at 40°C for 10 min. Non-fluorescently labeled ddNTP was purchased from Fermentas Inc. (Hanover, MD). All other labeling reagents were obtained from Amersham (Thermo Sequenase Dye Terminator Cycle Sequencing Core Kit, US 79565).

After labeling, each Streptawell was rinsed with 1X PBS (100 µl) three times. The "filled in" DNA fragments were then released from the Streptawells by digestion with the restriction enzyme EcoRI, according to the manufacturer's instructions that were supplied with the enzyme. Digestion was performed for 1 hour at 37 °C with shaking at 120 rpm.

Detection of the Locus of Interest

The samples were loaded into the lanes of a 36 cm 5% acrylamide (urea) gel (BioWhittaker Molecular Applications, Long Ranger Run Gel Packs, catalog number 50691). The samples were electrophoresed into the gel at 3000 volts for 3 min. The gel was run for 3 hours on a sequencing apparatus (Hoefer SQ3 Sequencer). The gel was removed from the apparatus and scanned on the Typhoon 9400 Variable Mode Imager. The incorporated labeled nucleotide was detected by fluorescence. A box was drawn around each band and the intensity of the band was calculated using the Typhoon 9400 Variable Mode Imager software.

Below, a schematic of the 5' overhang for SNP TSC0108992 is shown. The entire DNA sequence is not reproduced, only the portion to demonstrate the overhang (where R indicates the variable site).

					GTCC 3'
	G	A	C	R	CAGG 5'
	4	3	2	1	Overhang Position

The observed nucleotides for SNP TSC0108992 are adenine and thymidine on the sense strand (here depicted as the top strand). Position 3 of the overhang corresponds to adenine, which is complementary to thymidine. Labeled ddTTP was used in the presence of unlabeled dATP, dCTP, and dGTP. After the fill-in reaction with labeled ddTTP, the following DNA molecules were generated:

		T*	G	A	GTCC 3'	Allele 1
	G	A	C	T	CAGG 5'	
	4	3	2	1	Overhang Position	
				T*	GTCC 3'	Allele 2
	G	A	C	A	CAGG 5'	
	4	3	2	1	Overhang Position	

There was no difficulty in comparing the values obtained from allele 1 to allele 2 because one labeled nucleotide was used for the fill-in reaction, and the fill-in reaction for

both alleles occurred in a single tube. The alternate cutting properties of BsmF I would not influence this analysis because the 11/15 overhang would be filled in just as the 10/14 overhang. Schematics of the filled-in 11/15 overhangs are depicted below:

	T*	G	A	G	TCC 3' 11/15 Allele 1
	A	C	T	C	AGG 5'
	3	2	1	0	Overhang Position
			T*	G	TCC 3' 11/15 Allele 2
	A	C	A	C	AGG 5'
	3	2	1	0	Overhang Position

As seen in FIG. 16, two bands were seen for each sample of template DNA. The lower molecular weight band corresponded to the DNA molecules filled in with ddTTP at position one complementary to the overhang, and the higher molecular weight band corresponded to DNA molecules filled in with ddTTP at position 3 complementary to the overhang.

The percentage of allele 2 to allele 1 was highly consistent. (see Table XVII). In addition, for any given individual, the replicates of the PCR reaction showed similar results (see Table XVII). The percentage of allele 2 to allele 1 was calculated by dividing the value of allele 2 by the sum of the values for allele 1 and allele 2 (allele 2/(allele 1+ allele 2)). From four individuals, the average percentage of allele 2 to allele 1 was 0.4773 with a standard deviation of 0.0097. The percentage of allele 2 to allele 1 on template DNA isolated from an individual with Down's syndrome was 0.3086.

The theoretically expected percentage of allele 2 to allele 1 using template DNA from a normal individual is 0.50. However, the experimentally determined percentage was 0.4773. The theoretically expected percentage of allele 2 to allele 1 for an individual with an extra copy of chromosome 21 is 0.33. The experimentally determined percentage of allele 2 to allele 1 for SNP TSC0108992 was 0.3086.

The deviation from the theoretically expected percentage is highly consistent and remains linear. The following formula demonstrates that the percentage of allele 2 to allele 1 at SNP TSC0108992 remains linear even on template DNA obtained from an individual with an extra copy of chromosome 21:

$$\frac{0.47}{0.50} = \frac{X}{0.33}$$

$$X = 0.3102$$

If the percentage of allele 2 to allele 1 using template DNA obtained from a normal individual is determined to be 0.47, then the percentage of allele 2 to allele 1 using template DNA from an individual with Down's syndrome should be 0.3102. The experimentally determined ratio was 0.3086, with a standard deviation of 0.00186. There is no difference between the predicted percentage and the experimentally determined percentage of allele 2 to allele 1 on template DNA from an individual with Down's syndrome.

The percentage of one allele to the other allele at a particular SNP is highly consistent, reproducible, and linear. This demonstrates that any SNP, regardless of the calculated percentage for one allele to another, can be used to determine the presence or absence of a chromosomal disorder.

TABLE XVII. Percentage of Allele 2 to Allele 1 at SNP TSC0108992.

Sample	Allele 2	Allele 1	2/(2+1)
1A	9568886	10578972	0.474933
1B	8330864	9221381	0.474632
2A	9801053	10345444	0.486489
2B	8970942	9603102	0.482983
3A	8676718	9211085	0.485063
3B	10847024	11420943	0.487113
4A	10512420	12227107	0.462297
4B	7883584	9055289	0.465414
		MEAN	0.477366
		STDEV	0.009654

DS	6797400	15138959	0.309869
DS	6025753	13586890	0.307238
		MEAN	0.308554
		STDEV	0.00186

EXAMPLE 11

The percentage of allele 2 to allele 1 for a particular SNP is highly consistent. Statistically significant deviation from the experimentally determined ratio indicates the presence of a chromosomal abnormality. Below, the percentage of allele 2 to allele 1 at SNP TSC0108992 on chromosome 21 was calculated using template DNA from a normal individual and template DNA from an individual with Down's syndrome. Mixtures containing various amounts of normal DNA and Down's syndrome DNA were prepared and analyzed in a blind fashion.

Preparation of Template DNA

DNA was obtained from an individual with a normal genetic karyotype and an individual identified as having an extra copy of chromosome 21 (Down's syndrome). Informed consent was obtained from both individuals. Informed consent also was obtained from the parents of the individual with Down's syndrome.

From each individual, a 9 ml blood sample was collected into a sterile tube (Fischer Scientific, 9 ml EDTA Vacuette tubes, catalog number NC9897284). Template DNA was isolated using the QIAmp DNA Blood Midi Kit supplied by QIAGEN (Catalog number 51183). The template DNA was isolated as per instructions included in the kit.

Mixtures of Template DNA

The template DNA from the individual with the normal karyotype and the template DNA from the individual with an extra copy of chromosome 21 were diluted to a concentration of 10 ng/μl. Four mixtures of normal template DNA and Down's syndrome template DNA were made in the following fashion:

Mixture 1: 32 μl of Normal DNA + 8 μl of Down's syndrome DNA
Mixture 2: 28 μl of Normal DNA + 12 μl of Down's syndrome DNA
Mixture 3: 20 μl of Normal DNA + 20 μl of Down's syndrome DNA
Mixture 4: 10 μl of Normal DNA + 30 μl of Down's syndrome DNA

Three separate PCR reactions were set up for the normal template DNA and the template DNA from the individual with Down's syndrome. Likewise, for each mixture, three separate PCR reactions were set up.

Design of Primers

SNP TSC0108992 was amplified using the following primer set:

First primer:

5' CTACTGAGGGCTCGTAGATCCCAATTCCTTCCCAAGCT 3'

Second primer:

5' AATCCTGCTTTAGGGACCATGCTGGTGGA 3'

The first primer contained a biotin tag at the 5' end and a recognition site for the restriction enzyme EcoRI. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC0108992 was amplified from the template genomic DNA using the polymerase chain reaction (PCR, U.S. Patent Nos. 4,683,195 and 4,683,202, incorporated herein by reference). For increased specificity, a "hot-start" PCR was used. PCR reactions were performed using the HotStarTaq Master Mix Kit supplied by QIAGEN (catalog number 203443). The amount of template DNA and primer per reaction can be optimized for each locus of interest but in this example, 50 ng of template human genomic DNA and 5 μM of each primer were used. Thirty-eight cycles of PCR were performed. The following PCR conditions were used:

(1) 95°C for 15 minutes and 15 seconds;
(2) 37°C for 30 seconds;
(3) 95°C for 30 seconds;
(4) 57°C for 30 seconds;
(5) 95°C for 30 seconds;
(6) 64°C for 30 seconds;
(7) 95°C for 30 seconds;
(8) Repeat steps 6 and 7 thirty-seven (37) times;
(9) 72°C for 5 minutes.

In the first cycle of PCR, the annealing temperature was about the melting temperature of the 3′ annealing region of the second primers, which was 37°C. The annealing temperature in the second cycle of PCR was about the melting temperature of the 3' region, which anneals to the template DNA, of the first primer, which was 57°C. The annealing temperature in the third cycle of PCR was about the melting temperature of the entire sequence of the second primer, which was 64°C. The annealing temperature for the remaining cycles was 64°C. Escalating the annealing temperature from TM1 to TM2 to TM3 in the first three cycles of PCR greatly improves specificity. These annealing temperatures are representative, and the skilled artisan will understand the annealing temperatures for each cycle are dependent on the specific primers used.

The temperatures and times for denaturing, annealing, and extension, can be optimized by trying various settings and using the parameters that yield the best results.

Purification of Fragment of Interest

The PCR products were separated from the genomic template DNA. Each PCR reaction was split into two samples and transferred to two separate wells of a Streptawell, transparent, High-Bind plate from Roche Diagnostics GmbH (catalog number 1 645 692, as listed in Roche Molecular Biochemicals, 2001 Biochemicals Catalog). For each PCR reaction, there were two replicates, each in a separate well of a microtiter plate. The first primer contained a 5' biotin tag so the PCR products bound to the Streptavidin coated wells while the genomic template DNA did not. The streptavidin binding reaction was performed using a Thermomixer (Eppendorf) at 1000 rpm for 20 min. at 37°C. Each well was aspirated to remove unbound material, and washed three times with 1X PBS, with gentle mixing (Kandpal et al., Nucl. Acids Res. 18:1789-1795 (1990); Kaneoka et al., Biotechniques 10:30-34 (1991); Green et al., Nucl. Acids Res. 18:6163-6164 (1990)).

Restriction Enzyme Digestion of Isolated Fragments

The purified PCR products were digested with the restriction enzyme BsmF I, which binds to the recognition site incorporated into the PCR products from the second primer. The digests were performed in the Streptawells following the instructions supplied with the restriction enzyme. After digestion, the wells were washed three times with 1X PBS to remove the cleaved fragments.

Incorporation of Labeled Nucleotide

The restriction enzyme digest with BsmF I yielded a DNA fragment with a 5' overhang, which contained the SNP site or locus of interest and a 3' recessed end. The 5' overhang functioned as a template allowing incorporation of a nucleotide or nucleotides in the presence of a DNA polymerase.

As discussed in detail in Example 6, the sequence of both alleles of a SNP can be determined with one labeled nucleotide in the presence of the other unlabeled nucleotides. The following components were added to each fill in reaction: 1 µl of fluorescently labeled ddTTP, 0.5 µl of unlabeled ddNTPs (40 µM), which contained all nucleotides except thymidine, 2 µl of 10X sequenase buffer, 0.25 µl of Sequenase, and water as needed for a 20µl reaction. The fill in reaction was performed at 40°C for 10 min. Non-fluorescently labeled ddNTP was purchased from Fermentas Inc. (Hanover,

MD). All other labeling reagents were obtained from Amersham (Thermo Sequenase Dye Terminator Cycle Sequencing Core Kit, US 79565).

After labeling, each Streptawell was rinsed with 1X PBS (100 µl) three times. The "filled in" DNA fragments were then released from the Streptawells by digestion with the restriction enzyme EcoRI, according to the manufacturer's instructions that were supplied with the enzyme. Digestion was performed for 1 hour at 37 °C with shaking at 120 rpm.

Detection of the Locus of Interest

The samples were loaded into the lanes of a 36 cm 5% acrylamide (urea) gel (BioWhittaker Molecular Applications, Long Ranger Run Gel Packs, catalog number 50691). The samples were electrophoresed into the gel at 3000 volts for 3 min. The gel was run for 3 hours on a sequencing apparatus (Hoefer SQ3 Sequencer). The gel was removed from the apparatus and scanned on the Typhoon 9400 Variable Mode Imager. The incorporated labeled nucleotide was detected by fluorescence. A box was drawn around each band and the intensity of the band was calculated using the Typhoon 9400 Variable Mode Imager software.

As seen in FIGS. 17 A-F, two bands were seen. The lower molecular weight band corresponded to the DNA molecules filled in with ddTTP at position one complementary to the overhang. The higher molecular weight band corresponded to DNA molecules filled in with ddTTP at position 3 complementary to the overhang.

The experiment was performed in a blind fashion. The tubes were coded so that it was not known what tube corresponded to what template DNA. After the gels were analyzed, each tube was grouped into the following categories: normal template DNA, Down's syndrome template DNA, 3:1 mixture of Down's syndrome template DNA to normal DNA, 1:1 mixture of normal template DNA to Down's syndrome template DNA, 1:2.3 mixture of Down's syndrome template DNA to normal template DNA, and 1:4 mixture of Down's syndrome template DNA to normal template DNA. Each replicate of each PCR reaction successfully was grouped into the appropriate category, which demonstrates that the method can be used to detect abnormal DNA even if it represents only a small percentage of the total DNA.

The percentage of allele 2 to allele 1 for each replicate of the three PCR reactions from normal template DNA are displayed in Table XVIII (also see FIG. 17A). The average percentage of allele 2 to allele 1 was calculated by dividing the value of allele 2 by the sum of the values for allele 1 and allele 2 (allele 2 / (allele 1 + allele 2)), which resulted in an average of 0.50025 with a standard deviation of 0.002897. Thus, allele 1 and allele 2 were present in a ratio of 50:50. While the intensity of the bands varied from one PCR reaction to another (compare reaction 1 with reaction 3), there was no difference in intensity within a PCR reaction. Furthermore, the values obtained for the two replicates of the PCR reactions were very similar. Most of the variation was between PCR reactions and was likely attributable to pipetting errors.

The percentage of allele 2 to allele 1 for each replicate of the three PCR reactions from Down's syndrome template DNA are displayed in Table XVIII (see FIG. 17B). The percentage of allele 2 to allele 1 was calculated by dividing the value of allele 2 by the sum of the values for allele 1 and allele 2 (allele 2/allele 1+ allele 2), which resulted in an average of 0.301314 with a standard deviation of 0.012917. It is clear even upon analysis of the gel by the naked eye that allele 1 is present in a higher copy number than allele 2 (see FIG. 17B). Again, most of the variation occurs between PCR reactions and not within the replicate of a PCR reaction. The majority of the statistical variation likely resulted from pipetting errors.

Analysis of a single SNP was sufficient to detect the presence of the chromosomal abnormality. One SNP is sufficient provided that the "p" value of the SNP is known and that there are an adequate number of genomes so that statistical sampling error is not introduced into the analysis. In this experiment, there were approximately 5,000 genomes in each reaction.

The reactions that consisted of a mixture of Down's syndrome template DNA to normal template DNA at a ratio of 3:1 were clearly distinguishable from the normal template DNA, and the other mixtures of DNA (see FIG. 17C). The calculated percentage of allele 2 to allele 1 was 0.319089 with a standard deviation of 0.004346 (see Table XVIII). Likewise, the reactions that consisted of a mixture of Down's syndrome template DNA to normal template DNA at ratios of 1:1, and 1:2.3 were distinguishable (see FIG. 17D and 17E) and the values were statistically significant from all other reactions (see Table XVIII).

As the amount of normal template DNA increased, the percentage of allele 2 to allele 1 increased. With a mixture of Down's syndrome template DNA to normal template DNA of 1:4, the percentage of allele 2 to allele 1 was 0.397642, with a standard deviation of 0.001903 (see FIG 17F). The difference between this value and the value obtained from normal template DNA is statistically significant. Thus, the methods described herein allow the detection of a chromosomal abnormality even when the sample is not a homogeneous sample of abnormal DNA.

As described above, the presence of a small fraction of DNA with an abnormal copy number of chromosomes can be detected even among a large presence of normal DNA. It was clear, even by the naked eye, that as the amount of normal DNA increased and the amount of Down's syndrome DNA decreased, the intensities of the bands that corresponded to alleles 1 and 2 equalized.

The above example analyzed a SNP located on chromosome 21. However, any SNP may be analyzed on any chromosome including but not limited to human chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X, and Y and fetal chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X, and Y. In addition, chromosomes from non-human organisms can be analyzed using the above methods. Any combination of chromosomes can be analyzed. In the above example, an extra copy of a chromosome was detected. However, the same methods can be used to detect monosomies.

TABLE XVIII. Percentage of allele 2 to allele 1 at SNP TSC0108992 using normal template DNA and Down's syndrome template DNA.

		Normal Template DNA	
	Allele 1	**Allele 2**	**2/(2+1)**
1A	2602115	2604525	0.500231
1B	2855846	2923860	0.505884
2A	1954765	1941929	0.498353
2B	2084476	2068106	0.498029
3A	2044147	2035719	0.498967
3B	1760291	1760543	0.500036

		Mean	0.50025
		STD	0.002897
		Down's Syndrome	
	Allele 1	**Allele 2**	**2 /(2+1)**
1A	4046926	1595581	0.282779
1B	4275341	1736260	0.288818
2A	2875698	1299509	0.311244
2B	2453615	1069635	0.303593
3A	3169338	1426643	0.310411
3B	3737440	1687286	0.311036
		Mean	0.301314
		STD	0.012917
		3:1 (Down's: Normal)	
	Allele 1	**Allele 2**	**2/(2+1)**
1A	4067623	1980770	0.327487
1B	4058506	1899853	0.318855
2A	2315044	1085860	0.319286
2B	2686984	1243406	0.316357
3A	3880385	1790764	0.315767
3B	3718661	1724189	0.316781
		Mean	0.319089
		STD	0.004346
		1:1 (Down's: Normal)	
	Allele 1	**Allele 2**	**2/(2+1)**
1A	3540255	1929840	0.352798
1B	4004085	2161443	0.350569
2A	2358009	1282132	0.35222

2B	2158132	1238377	0.364603
3A	3052330	1648677	0.350707
3B	3852682	2024012	0.344413
		Mean	0.352552
		STD	0.006618
		1:2.3 (Down's: Normal)	
	Allele 1	**Allele 2**	**2/(2+1)**
1A	3109326	1942597	0.384526
1B	3392477	2118011	0.38436
2A	2824213	1758428	0.383715
2B	2069889	1249545	0.376433
3A	2335128	1433016	0.380298
3B	2916772	1797965	0.38135
		Mean	0.38178
		STD	0.003128
		1:4 (Down's: Normal)	
	Allele 1	**Allele 2**	**2/(2+1)**
1A	3066524	2039636	0.399446
1B	3068284	2038770	0.399207
2A	2325477	1542526	0.398791
2B	2366122	1562218	0.397679
3A	2151205	1403120	0.394764
3B	2397046	1571360	0.395968
		Mean	0.397642
		STD	0.001903

EXAMPLE 12

As discussed above in Example 9, the ratio for allele 1 to allele 2 at a heterozygous SNP is constant. However, one factor that can influence the ratio of allele 1 to allele 2 at a heterozygous SNP is a low number of genomes. For example, if there are 40 genomes, which means that there are a total of 40 chromosomes of allele 1 and 40 chromosomes of allele 2, it is statistically possible that the primers may anneal to 40 of the chromosomes with allele 1 but only 30 of the chromosomes with allele 2. This will affect the ratio of allele 1 to allele 2, and can erroneously influence the "p" value for a particular SNP.

Typically, whole genomic amplification, which employs degenerate oligonucleotide PCR, is used to increase low quantities of genomic DNA samples. Oligonucleotides of 8, 10, 12, or 14 bases are used to amplify the genome. It is thought that the primers anneal randomly throughout the genome, and will amplify a small genomic DNA sample into hundreds-fold more DNA for genetic analysis.

The methods described herein exploit the fact that typically the whole genome is not of interest. Particular loci of interest located on one chromosome, or on multiple chromosomes or on chromosomes that represent the entire genome are selected for analysis. Even if the loci of interest are located on chromosomes for the entire genome, it is preferential to amplify the region of those chromosomes that contain the loci of interest.

To overcome the limit of a low number of genomes, which is often seen with fetal DNA obtained from the plasma of a pregnant female, a multiplex method can be used to increase the number of genomes. The method described below preferentially amplifies the chromosome or chromosomes that contain the loci of interest.

Preparation of Template DNA

A 9 ml blood sample was collected into a sterile tube from a human volunteer after informed consent had been granted. (Fischer Scientific, 9 ml EDTA Vacuette tubes, catalog number NC9897284). The tubes were spun at 1000 rpm for ten minutes. The supernatant (the plasma) of each sample was removed, and one milliliter of the remaining blood sample, which is commonly referred to as the "buffy-coat" was transferred to a new tube. One milliliter of 1X PBS was added to each sample. Template DNA was

isolated using the QIAmp DNA Blood Midi Kit supplied by QIAGEN (Catalog number 51183).

Design of Multiplex Primers

Primers were designed to anneal at various regions on chromosome 21 to increase the copy number of the loci of interest located on chromosome 21. The primers were 12 bases in length. However, primers of any length can be used including but not limited to 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36-45, 46-55, 56-65, 66-75, 76-85, 86-95, 96-105, 106-115, 116-125, and greater than 125 bases. Primers were designed to anneal to both the sense strand and the antisense strand.

Nine SNPs located on chromosome 21 were analyzed: TSC0397235, TSC0470003, TSC1649726, TSC1261039, TSC0310507, TSC1650432, TSC1335008, TSC0128307, and TSC0259757. Any number of SNPs can be analyzed including but not limited to 1-10, 11-20, 21-30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90, 91-100, 101-200, 201-300, 301-400, 401-500, 501-600, 601-700, 701-800, 801-900, 901-1000, 1001-2000, 2001-3000, 3001-4000, 4001-5000, 5001-6000, 6001-7000, 7001-8000, 8001-9000, 9001-10,000 and greater than 10,000.

For each of the 9 SNPs, a 12 base primer was designed to anneal approximately 130 bases upstream of the loci of interest, and a 12 base primer was designed to anneal approximately 130 bases downstream of the loci of interest (herein referred to as the multiplex primers). The multiplex primers can be designed to anneal at any distance from the loci of interest including but not limited to 10-20, 21-30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90, 91-100, 101-110, 111-120, 121-130, 131-140, 141-150, 151-160, 161-170, 171-180, 181-190, 191-200, 201-210, 211-220, 221-230, 231-240, 241-250, 251-260, 261-270, 271-280, 281-290, 291-300, 301-310, 311-320, 321-330, 331-340, 341-350, 351-360, 361-370, 371-380, 381-390, 391-400, 401-410, 411-420, 421-430, 431-440, 441-450, 451-460, 461-470, 471-480, 481-490, 491-500, 501-600, 601-700, 701-800, 801-900, 901-1000, 1001-2000, 2001-3000, 3001-4000, 4001-5000, and greater than 5000 bases. In addition, more than one set of multiplex primers can be used for one SNP including but not limited to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 10-20, 21-30, 31-40, 41-50, and greater than 50.

In addition, 91 sets of forward and reverse primers were used to amplify other regions of chromosome 21, for a total of 100 sets of primers (200 primers in the reaction). These 91 primer sets were used to demonstrate that a large number of primers can be used in a single reaction without producing a large number of non-specific bands. Any number of primers can be used in the reaction including but not limited to 1-10, 11-20, 21-30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90, 91-100, 101-200, 201-300, 301-400, 401-500, 501-600, 601-700, 701-800, 801-900, 901-1000, 1001-2000, 2001-3000, 3001-4000, 4001-5000, 5001-6000, 6001-7000, 7001-8000, 8001-9000, 9001-10,000, 10,001-20,000, 20,001-30,000 and greater than 30,000.

The multiplex primers were designed to have the same nucleotides at the 3' end of the primer. In this case, the multiplex primers ended in "AA," wherein A indicates adenine. The primers were designed in this manner to minimize primer-dimer formation. However, the primers can terminate in any nucleotides including but not limited to adenine, guanine, cytosine, thymidine, any combination of adenine and guanine, any combination of adenine and cytosine, any combination of adenine and thymidine, any combination of guanine and cytosine, any combination of guanine and thymidine, or any combination of cytosine and thymidine. In addition the multiplex primers can have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more than 10 of the same nucleotides at the 3' end.

The multiplex primers for SNP TSC0397235 were:

Forward Primer:

5' CAAGTGTCCTAA 3'

Reverse primer:

5' CAGCTGCTAGAA 3'

The multiplex primers for SNP TSC0470003 were:

Forward Primer:

5' GGTTGAGGGCAA 3'

Reverse primer:

5' CACAGCGGGTAA 3'

The multiplex primers for SNP TSC1649726 were:

Forward Primer:

5' TTGACTTTTTAA 3'

Reverse primer:

5' ACAGAATGGGAA 3'

The multiplex primers for SNP TSC1261039 were:

Forward Primer:

5' TGCAGGTCACAA 3'

Reverse primer:

5' TTCTTCTTATAA 3'

The multiplex primers for SNP TSC0310507 were:

Forward Primer:

5' AGGACAACCTAA 3'

Reverse primer:

5' TGGTGTTCAGAA 3'

The multiplex primers for SNP TSC1650432 were:

Forward Primer:

5' TCAGCATATGAA 3'

Reverse primer:

5' GTTGCCACACAA 3'

The multiplex primers for SNP TSC1335008 were:

Forward Primer:

5' CCCAGCTAGCAA 3'

Reverse primer:

5' GGGTCACTGTAA 3'

The multiplex primers for SNP TSC0128307 were:

Forward Primer:

5' TTAAATACCCAA 3'

Reverse primer:

5' TTAGGAGGTTAA 3'

The multiplex primers for SNP TSC0259757 were:

Forward Primer:

5' ACACAGAATCAA 3'

Reverse primer:

5' CGCTGAGGTCAA 3'

Ninety-one (91) additional sets of primers, which annealed to various regions along chromosome 21, were included in the reaction:

Set 1:
Forward Primer:

5' AAGTAGAGTCAA 3'

Reverse primer:

5' CTTCCCATGGAA 3'

Set 2:
Forward Primer:

5' TTGGTTATTAAA 3'

Reverse primer:

5' CAACTTACTGAA 3'

Set 3:
Forward Primer:

5' CACTAAGTGAAA 3'

Reverse primer:

5' CTCACCTGCCAA 3'

Set 4:

Forward Primer:

5' ATGCATATATAA 3'

Reverse primer:

5' AGAGATCAGCAA 3'

Set 5:

Forward Primer:

5' TATATTTTTCAA 3'

Reverse primer:

5' CAGAAAGCAGAA 3'

Set 6:

Forward Primer:

5' GTATTGGGTTAA 3'

Reverse primer:

5' CTGACCCAGGAA 3'

Set 7:

Forward Primer:

5' CAGTTTTCCCAA 3'

Reverse primer:

5' AGGGCACAGGAA 3'

Set 8:

Forward Primer:

5' GTATCAGAGGAA 3'

Reverse primer:

5' GCATGAAAAGAA 3'

Set 9:

Forward Primer:

5' GATTTGACAGAA 3'

Reverse primer:

5' TACAGTTTACAA 3'

Set 10:

Forward Primer:

5' TGTGATTTTTAA 3'

Reverse primer:

5' TTATGTTCTCAA 3'

Set 11:

Forward Primer:

5' CAAGTACTTGAA 3'

Reverse primer:

5' CTTGTGTGGCAA 3'

Set 12:

Forward Primer:

5' AGACTTCTGCAA 3'

Reverse primer:

5' GTTGTCTTTCAA 3'

Set 13:

Forward Primer:

5' GGGACACTCCAA 3'

Reverse primer:

5' ATTATTATTCAA 3'

Set 14:

Forward Primer:

5' ACATGATGACAA 3'

Reverse primer:

5' TCAATTATAGAA 3'

Set 15:
Forward Primer:

5' CTATGGGCTGAA 3'

Reverse primer:

5' TGTGTGCCTGAA 3'

Set 16:
Forward Primer:

5' CCATTTGTTGAA 3'

Reverse primer:

5' TCTCCATCAAAA 3'

Set 17:
Forward Primer:

5' AATGCTGACAAA 3'

Reverse primer:

5' TTTCATGTCCAA 3'

Set 18:

Forward Primer:

5' GGCCTCTTGGAA 3'

Reverse primer:

5' TCATTTTTTGAA 3'

Set 19:

Forward Primer:

5' GGACTACCATAA 3'

Reverse primer:

5' AGTCACTCAGAA 3'

Set 20:

Forward Primer:

5' CCTTGGCAGGAA 3'

Reverse primer:

5' TTTCTGGTAGAA 3'

Set 21:

Forward Primer:

5' CCCCCCCCCGAA 3'

Reverse primer:

5' GCCCAGGCAGAA 3'

Set 22:

Forward Primer:

5' GAATGCGAAGAA 3'

Reverse primer:

5' TTAGGTAGAGAA 3'

Set 23:

Forward Primer:

5' TGCTTTGGTCAA 3'

Reverse primer:

5' GCCCATTAATAA 3'

Set 24:

Forward Primer:

5' TGAGATCTTTAA 3'

Reverse primer:

5' CAGTTTGTTCAA 3'

Set 25:

Forward Primer:

5' GCTGGGCAAGAA 3'

Reverse primer:

5' AGTCAAAGTCAA 3'

Set 26:
Forward Primer:

5' TCTCTGCAGTAA 3'

Reverse primer:

5' TGAATAACTTAA 3'

Set 27:
Forward Primer:

5' CGGTTAGAAAAA 3'

Reverse primer:

5' CATCCCTTTCAA 3'

Set 28:
Forward Primer:

5' TCTCTTTCTGAA 3'

Reverse primer:

5' CTCAGATTGTAA 3'

Set 29:

Forward Primer:

5' TTTGCACCAGAA 3'

Reverse primer:

5' GGTTAACATGAA 3'

Set 30:

Forward Primer:

5' ATTATCAACTAA 3'

Reverse primer:

5' GCCATTTTGTAA 3'

Set 31:

Forward Primer:

5' GATCTAGATGAA 3'

Reverse primer:

5' TTAATGTATTAA 3'

Set 32:

Forward Primer:

5' CTAGGGAGACAA 3'

Reverse primer:

5' TGGAGGAGACAA 3'

Set 33:

Forward Primer:

5' CATCACATTTAA 3'

Reverse primer:

5' GGGGTCCTGCAA 3'

Set 34:

Forward Primer:

5' CAGTTGTGCTAA 3'

Reverse primer:

5' TCTGCAGCCTAA 3'

Set 35:

Forward Primer:

5' GAGTCATTTAAA 3'

Reverse primer:

5' TCTATGGATTAA 3'

Set 36:

Forward Primer:

5' CAAAAAGTAGAA 3'

Reverse primer:

5' AATATACTCCAA 3'

Set 37:
Forward Primer:

5' CGTCCAGCACAA 3'

Reverse primer:

5' GGATGGTGAGAA 3'

Set 38:
Forward Primer:

5' TCTCCTTTGTAA 3'

Reverse primer:

5' TCGTTATTTCAA 3'

Set 39:
Forward Primer:

5' GATTTTATAGAA 3'

Reverse primer:

5' AGACATAAGCAA 3'

Set 40:

Forward Primer:

5' TTCACCTCACAA 3'

Reverse primer:

5' GGATTGCTTGAA 3'

Set 41:

Forward Primer:

5' ACTGCATGTGAA 3'

Reverse primer:

5' TTTATCACAGAA 3'

Set 42:

Forward Primer:

5' TCAGTAACACAA 3'

Reverse primer:

5' TACATCTTTGAA 3'

Set 43:

Forward Primer:

5' TTGTTTCAGTAA 3'

Reverse primer:

5' TATGAGCATCAA 3'

Set 44:

Forward Primer:

5' CTCAGCAGGCAA 3'

Reverse primer:

5' ACCCCTGTATAA 3'

Set 45:

Forward Primer:

5' TCTGCTCAGCAA 3'

Reverse primer:

5' GTTCTTTTTTAA 3'

Set 46:

Forward Primer:

5' GTGATAATCCAA 3'

Reverse primer:

5' GAGCCCTCAGAA 3'

Set 47:

Forward Primer:

5' TTTATTGGTTAA 3'

Reverse primer:

5' GGTACTGGGCAA 3'

Set 48:
Forward Primer:

5' AGTGTTTTTCAA 3'

Reverse primer:

5' TGTTATTGGTAA 3'

Set 49:
Forward Primer:

5' GCGCATTCACAA 3'

Reverse primer:

5' AAACAAAAGCAA 3'

Set 50:
Forward Primer:

5' TATATGATAGAA 3'

Reverse primer:

5' TCCCAGTTCCAA 3'

Set 51:

Forward Primer:

5' AAAGCCCATAAA 3'

Reverse primer:

5' TGTCATCCACAA 3'

Set 52:

Forward Primer:

5' TTGTGAATGCAA 3'

Reverse primer:

5' GTATTCATACAA 3'

Set 53:

Forward Primer:

5' TGACATAGGGAA 3'

Reverse primer:

5' AGCAAATTGCAA 3'

Set 54:

Forward Primer:

5' AGTAGATGTTAA 3'

Reverse primer:

5' AAAAGATAATAA 3'

Set 55:

Forward Primer:

5' ACCTCATGGGAA 3'

Reverse primer:

5' TGGTCGACCTAA 3'

Set 56:

Forward Primer:

5' TTTGCATGGTAA 3'

Reverse primer:

5' GCGGCTGCCGAA 3'

Set 57:

Forward Primer:

5' TCAGGAGTCTAA 3'

Reverse primer:

5' GCCTACCAGGAA 3'

Set 58:

Forward Primer:

5' ATCTTCTGTTAA 3'

Reverse primer:

5' AGGTAAGGACAA 3'

Set 59:
Forward Primer:

5' TGCTTTGAGGAA 3'

Reverse primer:

5' AACAGTTTTAAA 3'

Set 60:
Forward Primer:

5' TTAAATGTTTAA 3'

Reverse primer:

5' ATAGAAAATCAA 3'

Set 61:
Forward Primer:

5' GTGTTGTGTTAA 3'

Reverse primer:

5' GAGGACCTCGAA 3'

Set 62:

Forward Primer:

5' AGAGGCTGAGAA 3'

Reverse primer:

5' GGTATTTATTAA 3'

Set 63:

Forward Primer:

5' ATTTATCTGGAA 3'

Reverse primer:

5' AGTGCAAACTAA 3'

Set 64:

Forward Primer:

5' TGAACACCTTAA 3'

Reverse primer:

5' AATTTTTTCTAA 3'

Set 65:

Forward Primer:

5' TTACTATTATAA 3'

Reverse primer:

5' TGCTATAGTGAA 3'

Set 66:

Forward Primer:

5' TGGACTATGGAA 3'

Reverse primer:

5' CTGCAGTCCGAA 3'

Set 67:

Forward Primer:

5' GCTACTGCCCAA 3'

Reverse primer:

5' TCACATGGTGAA 3'

Set 68:

Forward Primer:

5' GTGGCTCTGGAA 3'

Reverse primer:

5' GAATTCCATTAA 3'

Set 69:

Forward Primer:

5' TGGGGTGTCCAA 3'

Reverse primer:

5' GCAAGCTCCGAA 3'

Set 70:
Forward Primer:

5' ATGTTTTTTCAA 3'

Reverse primer:

5' AGATCTGTTGAA 3'

Set 71:
Forward Primer:

5' AAGTGCTGTGAA 3'

Reverse primer:

5' ACTTTTTTGGAA 3'

Set 72:
Forward Primer:

5' AATCGGCAGGAA 3'

Reverse primer:

5' GGCATGTCACAA 3'

Set 73:

Forward Primer:

5' AGGAAGAAAGAA 3'

Reverse primer:

5' CAGTTTCACCAA 3'

Set 74:

Forward Primer:

5' CACAGAATTTAA 3'

Reverse primer:

5' AAGAATAAGTAA 3'

Set 75:

Forward Primer:

5' GGGATAGTACAA 3'

Reverse primer:

5' TTCCCATGATAA 3'

Set 76:

Forward Primer:

5' TGATTAGTTGAA 3'

Reverse primer:

5' GCATTCAGTGAA 3'

Set 77:

Forward Primer:

5' AGGGAATATTAA 3'

Reverse primer:

5' GACCTTAGGTAA 3'

Set 78:

Forward Primer:

5' TTCTTTTCACAA 3'

Reverse primer:

5' CCAAACTAAGAA 3'

Set 79:

Forward Primer:

5' GTGCTCTTAGAA 3'

Reverse primer:

5' ATGAGTTTAGAA 3'

Set 80:

Forward Primer:

5' ATGAGCATAGAA 3'

Reverse primer:

5' GACAAATGAGAA 3'

Set 81:
Forward Primer:

5' AAACCCAGAGAA 3'

Reverse primer:

5' CCTCACACAGAA 3'

Set 82:
Forward Primer:

5' CACACTGTGGAA 3'

Reverse primer:

5' CACTGTACCCAA 3'

Set 83:
Forward Primer:

5' GTAGTATTTCAA 3'

Reverse primer:

5' TGGATACACTAA 3'

Set 84:

Forward Primer:

5' CCCATGATTCAA 3'

Reverse primer:

5' TCATAGGAGGAA 3'

Set 85:

Forward Primer:

5' AGGAAAGAGAAA 3'

Reverse primer:

5' ATATGGTGATAA 3'

Set 86:

Forward Primer:

5' GATGCCATCCAA 3'

Reverse primer:

5' ATACTATTTCAA 3'

Set 87:

Forward Primer:

5' GTGTGCATGGAA 3'

Reverse primer:

5' AGGTGTTGAGAA 3'

Set 88:

Forward Primer:

5' CAGCCTGGGCAA 3'

Reverse primer:

5' GGAGCTCTACAA 3'

Set 89:

Forward Primer:

5' AACTAAGGTTAA 3'

Reverse primer:

5' AACTTATGTTAA 3'

Set 90:

Forward Primer:

5' ATCTCAACAGAA 3'

Reverse primer:

5' TAACAATGTGAA 3'

Set 91:

Forward Primer:

5' AAGGATCAGGAA 3'

Reverse primer:

5' CTCAAGTCTTAA 3'

Multiplex PCR

Regions on chromosome 21 surrounding SNPs TSC0397235, TSC0470003, TSC1649726, TSC1261039, TSC0310507, TSC1650432, TSC1335008, TSC0128307, and TSC0259757 were amplified from the template genomic DNA using the polymerase chain reaction (PCR, U.S. Patent Nos. 4,683,195 and 4,683,202, incorporated herein by reference). This PCR reaction used primers that annealed approximately 130 bases upstream and downstream of the loci of interest. It was used to increases the number of copies of the loci of interest to eliminate any errors that may result from a low number of genomes.

For increased specificity, a "hot-start" PCR reaction was used. PCR reactions were performed using the HotStarTaq Master Mix Kit supplied by QIAGEN (catalog number 203443). The amount of template DNA and primer per reaction can be optimized for each locus of interest. In this example, 15 ng of template human genomic DNA and 5 μM of each primer were used.

Two microliters of each forward and reverse primer, at concentrations of 5 mM were pooled into a single microcentrifuge tube and mixed. Eight microliters of the primer mix was used in a total PCR reaction volume of 40 μl (1.5 μl of template DNA, 10.5 μl of sterile water, 8 μl of primer mix, and 20 μl of HotStar Taq). Twenty-five cycles of PCR were performed. The following PCR conditions were used:

(1) 95°C for 15 minutes;

(2) 95°C for 30 seconds;

(3) 4°C for 30 seconds;

(4) 37°C for 30 seconds;

(5) Repeat steps 2-4 twenty-four (24) times;

(6) 72°C for 10 minutes.

The temperatures and times for denaturing, annealing, and extension, can be optimized by trying various settings and using the parameters that yield the best results.

Purification of Fragment of Interest

The excess primers and nucleotides were removed from the reaction by using Qiagen MinElute PCR purification kits (Qiagen, Catalog Number 28004). The reactions were performed following the manufacturer's instructions supplied with the columns. The DNA was eluted in 100 µl of sterile water.

PCR Reaction Two

SNP TSC0397235 was amplified using the following primer set:

First Primer:

5' TTAGTCATCGCAGAATTCTACTTCTTTCTGAAGTGGGA 3'

Second primer:

5' GGACAGCTCGATGGGACTAATGCATACTC 3'

The first primer contained a biotin tag at the 5' end and a recognition site for the restriction enzyme EcoRI, and was designed to anneal 103 bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC0470003 was amplified using the following primer set:

First Primer:

5' GTAGCCACTGGTGAATTCGTGCCATCGCAAAAGAATAA 3'

Second primer:

5' ATTAGAATGATGGGGACCCCTGTCTTCCC 3'

The first primer contained a biotin tag at the 5' end and a recognition site for the restriction enzyme EcoRI, and was designed to anneal 80 bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC1649726 was amplified using the following primer set:

First Primer:

5' ACGCATAGGAAGGAATTCATTCTGACACGTGTGAGATA 3'

Second primer:

5' GAAATTGACCACGGGACTGCACACTTTTC 3'

The first primer contained a biotin tag at the 5' end and a recognition site for the restriction enzyme EcoRI, and was designed to anneal 113 bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC1261039 was amplified using the following primer set:

First Primer:

5' CGGTAAATCGGAGAATTCAAGTTGAGGCATGCATCCAT 3'

Second primer:

5' TCGGGGCTCAGCGGGACCACAGCCACTCC 3'

The first primer contained a biotin tag at the 5' end and a recognition site for the restriction enzyme EcoRI, and was designed to anneal 54 bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC0310507 was amplified using the following primer set:

First Primer:

5' TCTATGCACCACGAATTCAATATGTGTTCAAGGACATT 3'

Second primer:

5' TGCTTAATCGGTGGGACTTGTAATTGTAC 3'

The first primer contained a biotin tag at the 5' end and a recognition site for the restriction enzyme EcoRI, and was designed to anneal 93 bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC1650432 was amplified using the following primer set:

First Primer:

5' CGCGTTGTATGCGAATTCCCTGGGGTATAAAGATAAGA 3'

Second primer:

5' CTCACGGGAACTGGGACACCTGACCCTGC 3'

The first primer contained a biotin tag at the 5' end and a recognition site for the restriction enzyme EcoRI, and was designed to anneal 80 bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC1335008 was amplified using the following primer set:

First Primer:

5' GTCTTGCCGCTTGAATTCCCATAGAAGAATGCGCCAAA 3'

Second primer:

5' TTGAGTAGTACAGGGACACACTAACAGAC 3'

The first primer contained a biotin tag at the 5' end and a recognition site for the restriction enzyme EcoRI, and was designed to anneal 94 bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC0128307 was amplified using the following primer set:

First Primer:

5' AATACTGTAGGTGAATTCTTGCCTAAGCATTTTCCCAG 3'

Second primer:

5' GTGTTGACATTCGGGACTGTAATCTTGAC 3'

The first primer contained a biotin tag at the 5' end and a recognition site for the restriction enzyme EcoRI, and was designed to anneal 54 bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

SNP TSC0259757 was amplified using the following primer set:

First Primer:

5' TCTGTAGATTCGGAATTCTTTAGAGCCTGTGCGCTGAG 3'

Second primer:

5' CGTACCAGTACAGGGACGCAAACTGAGAC 3'

The first primer contained a biotin tag at the 5' end and a recognition site for the restriction enzyme EcoRI, and was designed to anneal 100 bases from the locus of interest. The second primer contained the recognition site for the restriction enzyme BsmF I.

All loci of interest were amplified from the template genomic DNA using the polymerase chain reaction (PCR, U.S. Patent Nos. 4,683,195 and 4,683,202, incorporated herein by reference). In this example, the loci of interest were amplified in separate reaction tubes but they can also be amplified together in a single PCR reaction. For increased specificity, a "hot-start" PCR was used. PCR reactions were performed using the HotStarTaq Master Mix Kit supplied by QIAGEN (catalog number 203443).

One microliter of the elutate from the multiplex reaction (PCR product eluted from the MinElute column) was used as template DNA for each PCR reaction. Each SNP was amplified in triplicate when the multiplex sample was used as the template. As a control, each SNP was amplified from 15 ng of the original template DNA (DNA that did not undergo the multiplex reaction). The amount of template DNA and primer per reaction can be optimized for each locus of interest but in this example, 5 μM of each primer was used. Forty cycles of PCR were performed. The following PCR conditions were used:

(1) 95°C for 15 minutes and 15 seconds;

(2) 37°C for 30 seconds;

(3) 95°C for 30 seconds;

(4) 57°C for 30 seconds;

(5) 95°C for 30 seconds;

(6) 64°C for 30 seconds;

(7) 95°C for 30 seconds;

(8) Repeat steps 6 and 7 thirty nine (39) times;

(9) 72°C for 5 minutes.

In the first cycle of PCR, the annealing temperature was about the melting temperature of the 3′ annealing region of the second primers, which was 37°C. The annealing temperature in the second cycle of PCR was about the melting temperature of the 3' region, which anneals to the template DNA, of the first primer, which was 57°C. The annealing temperature in the third cycle of PCR was about the melting temperature of the entire sequence of the second primer, which was 64°C. The annealing temperature for the remaining cycles was 64°C. Escalating the annealing temperature from TM1 to TM2 to TM3 in the first three cycles of PCR greatly improves specificity. These annealing temperatures are representative, and the skilled artisan will understand the annealing temperatures for each cycle are dependent on the specific primers used.

The temperatures and times for denaturing, annealing, and extension, can be optimized by trying various settings and using the parameters that yield the best results.

Agarose Gel Analysis

Four microliters of a twenty microliter PCR reaction for each SNP from the original template DNA was analyzed by agarose gel electrophoresis (see FIG. 18A). Four microliters of a twenty microliter PCR reaction for each SNP that was amplified from the multiplexed template was analyzed on by agarose gel electrophoresis (see FIG. 18B).

As seen in FIG. 18A, for 8/9 of the SNPs amplified from the original template DNA, a single band of high intensity was seen (lanes 1-3, and 5-9). The band migrated at the correct position for each of the 8 SNPs. Amplification of TSC1261039 from the original template DNA produced a band of high intensity, which migrated at the correct

position, and a faint band of lower molecular weight (lane 4). Only two bands were seen, and the bands could clearly be distinguished based on molecular weight. The PCR method described herein allows clean amplification of the loci of interest from genomic DNA without any concentration or enrichment of the loci of interest.

As seen in FIG. 18B, the primers used to amplify SNPs TSC0397235, TSC0470003, TSC0310507, and TSC0128307 from the multiplexed template DNA produced a single band of high intensity, which migrated at the correct position (lanes 1, 2, 5, and 8). No additional bands were introduced despite the fact that the multiplex reaction contained two hundred primers. While the multiplex primers were 12 bases in length and likely annealed to additional sequences other than those located on chromosome 21, the products were not seen because the bands were not amplified in the second PCR reaction. The second PCR reaction employed primers specific for the loci of interest and used asymmetric oligonucleotides and escalating annealing temperatures, which allows specific amplification from the genome (see Example 1).

Amplification of TSC1649726 from the multiplex template DNA produced one band of high intensity and two weaker bands, which could clearly be distinguished based on molecular weight (see FIG. 18B, lane 3). Amplification of TSC1261039 from the multiplex template DNA produced a high intensity band of the correct molecular weight and a faint band of lower molecular weight (see FIG. 18B, lane 4). The low molecular weight band was the same size as the band seen from the amplification of TSC1261039 from the original template DNA (compare FIG. 18A, lane 4 with FIG. 18B, lane 4). Thus, amplification of TSC1261039 on the multiplex template DNA did not introduce any additional non-specific bands

Amplification of SNPs TSC1650432, TSC1335008, and TSC0259757 from the multiplex template DNA produced one band of high intensity, which migrated at the correct position, and one weaker band (lanes 6, 7, and 9). For SNPs TSC1650432 and TSC0259757, the weaker band was of lower molecular weight, and clearly was distinguishable from the band of interest (see FIG. 18B, lanes 6 and 9). For SNP TSC1335008, the weaker band was of slightly higher molecular weight. However, the correct band can be identified by comparing to the amplification products of TSC1335008 from the original template DNA, (compare FIG. 18A, lane 7 and FIG. 18B, lane 7). The PCR conditions can also be optimized for TSC1335008. All 9 SNPs were

amplified under the exact same conditions, which produced clearly distinguishable bands for the amplified SNPs.

Purification of Fragment of Interest

The PCR products were separated from the genomic template DNA. One half of the PCR reaction was transferred to a well of a Streptawell, transparent, High-Bind plate from Roche Diagnostics GmbH (catalog number 1 645 692, as listed in Roche Molecular Biochemicals, 2001 Biochemicals Catalog). The first primers contained a 5' biotin tag so the PCR products bound to the Streptavidin coated wells while the genomic template DNA did not. The streptavidin binding reaction was performed using a Thermomixer (Eppendorf) at 1000 rpm for 20 min. at 37°C. Each well was aspirated to remove unbound material, and washed three times with 1X PBS, with gentle mixing (Kandpal et al., Nucl. Acids Res. 18:1789-1795 (1990); Kaneoka et al., Biotechniques 10:30-34 (1991); Green et al., Nucl. Acids Res. 18:6163-6164 (1990)).

Restriction Enzyme Digestion of Isolated Fragments

The purified PCR products were digested with the restriction enzyme BsmF I, which binds to the recognition site incorporated into the PCR products from the second primer. The digests were performed in the Streptawells following the instructions supplied with the restriction enzyme. After digestion, the wells were washed three times with PBS to remove the cleaved fragments.

Incorporation of Labeled Nucleotide

The restriction enzyme digest with BsmF I yielded a DNA fragment with a 5' overhang, which contained the SNP site or locus of interest and a 3' recessed end. The 5' overhang functioned as a template allowing incorporation of a nucleotide or nucleotides in the presence of a DNA polymerase.

As discussed in detail in Example 6, the sequence of both alleles of a SNP can be determined by using one labeled nucleotide in the presence of the other unlabeled nucleotides. The following components were added to each fill in reaction: 1 µl of

fluorescently labeled ddGTP, 0.5 µl of unlabeled ddNTPs (40 µM), which contained all nucleotides except guanine, 2 µl of 10X sequenase buffer, 0.25 µl of Sequenase, and water as needed for a 20µl reaction. The fill in reaction was performed at 40°C for 10 min. Non-fluorescently labeled ddNTP was purchased from Fermentas Inc. (Hanover, MD). All other labeling reagents were obtained from Amersham (Thermo Sequenase Dye Terminator Cycle Sequencing Core Kit, US 79565).

After labeling, each Streptawell was rinsed with 1X PBS (100 µl) three times. The "filled in" DNA fragments then were released from the Streptawells by digestion with the restriction enzyme EcoRI, according to the manufacturer's instructions that were supplied with the enzyme. Digestion was performed for 1 hour at 37 °C with shaking at 120 rpm.

Detection of the Locus of Interest

The samples were loaded into a lane of a 36 cm 5% acrylamide (urea) gel (BioWhittaker Molecular Applications, Long Ranger Run Gel Packs, catalog number 50691). The samples were electrophoresed into the gel at 3000 volts for 3 min. The gel was run for 3 hours on a sequencing apparatus (Hoefer SQ3 Sequencer). The gel was removed from the apparatus and scanned on the Typhoon 9400 Variable Mode Imager. The incorporated labeled nucleotide was detected by fluorescence. A box was drawn around each band and the intensity of the band was calculated using the ImageQuant software.

Below, a schematic of the 5' overhang for TSC0470003 after digestion with BsmF I is depicted:

	5' CTCT				
	3' GAGA	R	A	C	C
Overhang position		1	2	3	4

The observed nucleotides for TSC0470003 are adenine and guanine on the sense strand (herein depicted as the top strand). The third position of the overhang corresponds to cytosine, which is complementary to guanine. Labeled ddGTP was used in the

presence of unlabeled dATP, dCTP, and dTTP. Schematics of the DNA molecules after the fill-in reaction are depicted below:

Allele 1	5' CTCT	G*			
	3' GAGA	C	A	C	C
Overhang position		1	2	3	4
Allele 2	5' CTCT	A	T	G*	
	3' GAGA	T	A	C	C
Overhang position		1	2	3	4

Two bands were seen; the lower molecular weight band corresponded to the DNA molecules filled in with ddGTP at position 1 complementary to the overhang and the higher molecular weight band corresponded to the DNA molecules filled in with ddGTP at position 3 complementary to the overhang (see FIG. 19).

The percentage of allele 2 to allele 1 at TSC0470003 after amplification from the original template DNA and the multiplexed template DNA was calculated. The use of one fluorescently labeled nucleotide to detect both alleles in a single reaction reduces the amount of error that is introduced through pipetting reactions, and the error that is introduced through the quantum coefficients of different dyes.

For SNP TSC047003, the percentage of allele 2 to allele 1 was calculated by dividing the value of allele 2 by the sum of the values for allele 2 and allele 1. The percentage of allele 2 to allele 1 for TSC047003 on the original template DNA was calculated to be 0.539 (see Table XIX). Three PCR reactions were performed for each SNP on the multiplexed template DNA. The average percentage of allele 2 to allele 1 for TSC047003 on the multiplexed DNA was 0.49 with a standard deviation of 0.0319 (see Table XIX). There was no statistically significant difference between the percentage obtained on the original template DNA and the multiplexed template DNA.

For SNP TSC1261039, the percentage of allele 2 to allele 1 for TSC1261039 on the original template DNA was calculated to be 0.44 (see Table XIX). Three PCR reactions were performed for each SNP on the multiplexed template DNA (see FIG. 19B). The average percentage of allele 2 to allele 1 for TSC1261039 on the multiplexed DNA was 0.468 with a standard deviation of 0.05683 (see Table XIX). There was no

statistically significant difference between the percentages of allele 2 to allele 1 obtained on the original template DNA and the multiplexed template DNA.

The variation seen in the percentage of allele 2 to allele 1 for TSC1261039 on the multiplexed template DNA was likely due to pipetting reactions. The variation can be reduced by increasing the number of replicates. With a large number of replicates, a percentage can be obtained with minimum statistical variation.

Likewise, there was no statistical difference between the percentage of allele 2 to allele 1 on the original template DNA and on the multiplexed template DNA for SNPs TSC0310507 and TSC1335008 (see Table XIX, and FIGS. 19C and 19D). Thus, a multiplex reaction can be used to increase the number of chromosomal regions containing the loci of interest without affecting the percentage of one allele to the other at the variable sites.

TABLE XIX. Percentage of allele 2 to allele 1 at various SNPs with and without multiplexing.

TSC047003			
	Allele 1	**Allele 2**	**2/(2+1)**
IA	5535418	6487873	0.539608748
M1	4804358	4886716	0.504249168
M2	5549389	5958585	0.517778803
M3	8356275	7030245	0.45690936
Mean (M1-M3)			0.49297911
STDEV			0.031961429
TSC1261039			
	Allele 1	**Allele 2**	**2/(2+1)**
IA	3488765	2768066	0.442407027
M1	3603388	2573244	0.41660957
M2	4470423	5026872	0.529295131

M3	4306015	36694012	0.46008898
Mean (M1-M3)			0.46866456
STDEV			0.056830136
TSC0310507			
	Allele 1	**Allele 2**	**2/(2+1)**
IA	2966511	2688190	0.475390299
M1	4084472	2963451	0.420471535
M2	4509891	4052892	0.47331481
M3	7173191	4642069	0.39288759
Mean (M1-M3)			0.428891312
STDEV			0.040869352
TSC1335008			
	Allele 1	**Allele 2**	**2/(2+1)**
IA	2311629	2553016	0.524810341
M1	794790	900879	0.531282343
M2	1261568	1780689	0.5853184
M3	1165156	1427840	0.550653
Mean (M1-M3)			0.555751248
STDEV			0.027376412

The methods described herein used two distinct amplification reactions to amplify the loci of interest. In the first PCR reaction, oligonucleotides were designed to anneal upstream and downstream of the loci of interest. Unlike traditional genomic amplification, these primers were not degenerate and annealed at a specified distance from the loci of interest. However, due to the length of the primers, it is likely that the

primers annealed to other regions of the genome. These primers were used to increase the amount of DNA available for genetic analysis.

The second PCR reaction employs the methods described in Examples 1-6. The primers are designed to amplify the loci of interest, and the sequence is determined at the loci of interest. The conditions of the second PCR reaction allowed specific amplification of the loci of interest from the multiplexed template DNA. If there were any non-specific products from the multiplex reaction, they did not impede amplification of the loci of interest. There was no statistical difference in the percentages of allele 2 to allele 1 at the four SNPs analyzed, regardless of whether the amplification was performed on original template DNA or multiplexed template DNA.

The SNPs analyzed in this example were located on human chromosome 21. However, the methods can be applied to non-human and human DNA including but not limited to chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X, and Y. The multiplex methods can also be applied to analysis of genetic mutations including but not limited to nucleotide substitutions, insertions, deletions, and rearrangements.

The above methods can be used to increase the amount of DNA available for genetic analysis whenever the starting template DNA is limiting in quantity. For example, premalignant and preinvasive lesions with malignant cells usually constitute a small fraction of the cells in the specimen, which reduces the number of genetic analyses that can be performed. The methods described herein can be used to increase the amounts of malignant DNA available for genetic analysis. Also, the number of fetal genomes present in the maternal blood is often low; the methods described herein can be used to increase the amount of fetal DNA.

EXAMPLE 13

Plasma isolated from blood of a pregnant female contains both maternal template DNA and fetal template DNA. As discussed earlier, the percentage of fetal DNA in the maternal plasma varies for each pregnant female. However, the percentage of fetal DNA can be determined by analyzing SNPs wherein the maternal template DNA is homozygous and the template DNA obtained from the plasma displays a heterozygous pattern.

For example, assume SNP X can either be adenine or guanine, and the maternal DNA for SNP X is homozygous for guanine. The labeling method described in Example 6 can be used to determine the sequence of the template DNA in the plasma sample. If the plasma sample contains fetal DNA, which is heterozygous at SNP X, the following DNA molecules are expected after digestion with the type IIS restriction enzyme BsmF I, and the fill-in reaction with labeled ddGTP, unlabeled dATP, dTTP, and dCTP.

Maternal Allele 1	5' GGGT	**G***			
	3'CCCA	C	T	C	A
Maternal Allele 2	5' GGGT	**G***			
	3'CCCA	C	T	C	A
Fetal Allele 1	5' GGGT	**G***			
	3'CCCA	C	T	C	A
Fetal Allele 2	5' GGGT	**A**	**A**	**G***	
	3'CCCA	T	T	C	A

Two signals are seen; one signal corresponds to the DNA molecules filled in with ddGTP at position one complementary to the overhang and the second signal corresponds to the DNA molecules filled in with ddGTP at position three complementary to the overhang. However, the maternal DNA is homozygous for guanine, which corresponds to the DNA molecules filled in at position one complementary to the overhang. The signal from the DNA molecules filled in with ddGTP at position three complementary to the overhang corresponds to the adenine allele, which represents the fetal DNA. This signal becomes a beacon for the fetal DNA, and can used to measure the amount of fetal DNA present in the plasma sample.

There is no difference in the amount of fetal DNA from one chromosome to another. For instance, the percentage of fetal DNA in any given individual from chromosome 1 is the same as the percentage of fetal DNA from chromosome 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X and Y. Thus, the allele ratio

calculated for SNPs on one chromosome can be compared to the allele ratio for the SNPs on another chromosome.

For example, the allele ratio for the SNPs on chromosome 1 should be equal to the allele ratio for the SNPs on chromosomes 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X, and Y. However, if the fetus has a chromosomal abnormality, including but not limited to a trisomy or monosomy, the ratio for the chromosome that is present in an abnormal copy number will differ from the ratio for the other chromosomes.

Blood from a pregnant female was collected after informed consent had been obtained. The blood sample was used to demonstrate that fetal DNA can be detected in the maternal plasma by analyzing SNPs wherein the maternal DNA was homozygous, and the same SNP displayed a heterozygous pattern from DNA obtained from the plasma of a pregnant woman.

Preparation of Plasma from Whole Blood

Plasma was isolated from 4 tubes each containing 9 ml of blood (Fischer Scientific, 9 ml EDTA Vacuette tubes, catalog number NC9897284). The blood was obtained by venipuncture from a pregnant female who had given informed consent. After collecting the blood, formaldehyde (25 µl/ml of blood) was added to each of the tubes. The tubes were placed at 4°C until shipment. The tubes were shipped via Federal Express in a foam container containing an ice pack.

The blood was centrifuged at 1000 rpm for 10 minutes. The brake on the centrifuge was not used. This centrifugation step was repeated. The supernatant was transferred to a new tube and spun at 3,000 rpm for ten minutes. The brake on the centrifuge was not used. The supernatant from each of the four tubes was pooled and aliquoted into two tubes. The plasma was stored at -80°C until the DNA was purified.

Template DNA was isolated using the QIAmp DNA Blood Midi Kit supplied by QIAGEN (Catalog number 51183). The template DNA was isolated as per instructions included in the kit. The template DNA from the plasma was eluted in a final volume of 20 microliters.

Isolation of Maternal DNA

After the plasma was removed from the sample described above, one milliliter of the remaining blood sample, which is commonly referred to as the "buffy-coat," was transferred to a new tube. One milliliter of 1X PBS was added to the sample. Template DNA was isolated using the QIAmp DNA Blood Midi Kit supplied by QIAGEN (Catalog number 51183).

Identification of Homozygous Maternal SNPs

Example 8 describes a method for identifying SNPs that are highly variable within the population or for identifying heterozygous SNPs for a given individual. The methods as described in Example 8 were applied to the maternal template DNA to identify SNPs on chromosome 13 wherein the maternal DNA was homozygous. Any number of SNPs can be screened. The number of SNPs to be screened is proportional to the number of heterozygous SNPs in the fetal DNA that need to be analyzed.

As described in detail in Example 6, one labeled nucleotide can be used to determine the sequence of both alleles at a particular SNP. SNPs for which the sequence can be determined with labeled ddGTP in the presence of unlabeled dATP, dTTP, and dCTP were chosen for this example. However, SNPs for which the sequence can be determined with labeled ddATP, ddCTP or ddTTP can also be used. Additionally, the SNPs to be analyzed can be chosen such that all are labeled with the same nucleotide or any combination of the four nucleotides. For instance, if 400 SNPs are to be screened, 100 can be chosen such that the sequence is determined with labeled ddATP, 100 can be chosen such that the sequence is determined with labeled ddTTP, 100 can be chosen such that the sequence is determined with labeled ddGTP, and 100 can be chosen such that the sequence is determined with labeled ddCTP, or any combination of the four labeled nucleotides.

Twenty-nine SNPs wherein the maternal DNA was homozygous were identified: TSC0052277, TSC1225391, TSC0289078, TSC1349804, TSC0870209, TSC0194938, TSC0820373, TSC0902859, TSC0501510, TSC1228234, TSC0082910, TSC0838335, TSC0818982, TSC0469204, TSC1084457, TSC0466177, TSC1270598, TSC1002017, TSC1104200, TSC0501389, TSC0039960, TSC0418134, TSC0603688, TSC0129188,

TSC1103570, TSC0813449, TSC0701940, TSC0087962, and TSC0660274. Heterozygous SNPs will vary from individual to individual.

Design of Multiplex Primers

A low copy number of fetal genomes typically is present in the maternal plasma. To increase the copy number of the loci of interest located on chromosome 13, primers were designed to anneal at approximately 130 bases upstream and 130 bases downstream of each loci of interest. This was done to reduce statistical sampling error that can occur when working with a low number of genomes, which can influence the ratio of one allele to another (see Example 11). The primers were 12 bases in length. However, primers of any length can be used including but not limited to 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36-45, 46-55, 56-65, 66-75, 76-85, 86-95, 96-105, 106-115, 116-125, and greater than 125 bases. Primers were designed to anneal to both the sense strand and the antisense strand.

The primers were designed to terminate at the 3' end in the dinucleotide "AA" to reduce the formation of primer-dimers. However, the primers can be designed to end in any of the four nucleotides and in any combination of the four nucleotides.

The multiplex primers for SNPTSC0052277 were

Forward primer:

5' GACATGTTGGAA 3'

Reverse primer:

5' ACTTCCAGTTAA 3'

The multiplex primers for SNP TSC1225391 were:

Forward primer:

5' GTTTCCTGTTAA 3'

Reverse primer

5' CGATGATGACAA 3'

The multiplex primers for SNP TSC0289078 were:

Forward primer

5' GAGTAGAGACAA 3'

Reverse primer

5' TCCCGGATACAA 3'

The multiplex primes for SNP TSC1349804 were:

Forward primer:

5' CATCCTCTAGAA 3'

Reverse primer:

5' TATTCCTGAGAA 3'

The multiplex primers for SNP TSC0870209 were:

Forward primer:

5' AGTTTGTTTTAA 3'

Reverse primer:

5' TATAAACGATAA 3'

The multiplex primers for SNP TSC0194938 were:

Forward primer:

5' TTTGACCGATAA 3'

Reverse primer:

5' TGACAGGACCAA 3'

The multiplex primers for SNP TSC0820373 were:

Forward primer:

5' TTATTCATTCAA 3'

Reverse primer:

5' AGTTTTTCACAA 3'

The multiplex primers for SNP TSC0902859 were:

Forward primer:

5' CACCTCCCTGAA 3'

Reverse primer:

5' CCAGATTGAGAA 3'

The multiplex primers for SNP TSC0501510 were:

Forward primer:

5' TGTGTCCACCAA 3'

Reverse primer:

5' CTTCTATTCCAA 3'

The multiplex primers for SNP TSC1228234 were:

Forward primer:

5' TCACAATAGGAA 3'

Reverse primer:

5' TACAAGTGAGAA 3'

The multiplex primers for SNP TSC0082910 were:

Forward primer:

5' GAGTTTTCGTAA 3'

Reverse primer:

5' GTGTGCCCCCAA 3'

The multiplex primers for SNP TSC0838335 were:

Forward primer:

5' GCACCACTGCAA 3'

Reverse primer:

5' GAACACAATGAA 3'

The multiplex primers for SNP TSC0818982 were:

Forward primer:

5' TATCCTATTCAA 3'

Reverse primer:

5' CAACCATTATAA 3'

The multiplex primers for SNP TSC0469204 were:

Forward primer:

5' TATGCTTTACAA 3'

Reverse primer:

5' TTTGTTTACCAA 3'

The multiplex primers for SNP TSC1084457 were:

Forward primer:

5' AGGAAATTAGAA 3'

Reverse primer:

5' TGTTAGACTTAA 3'

The multiplex primers for SNP TSC0466177 were:

Forward primer:

5' TATTTGGAGGAA 3'

Reverse primer:

5' GGCATTTGTCAA 3'

The multiplex primers for SNP TSC1270598 were:

Forward primer:

5' ATACTCCAGGAA 3'

Reverse primer:

5' CAGCCTGGACAA 3'

The multiplex primers for SNP TSC1002017 were:

Forward primer:

5' CCATTGCAGTAA 3'

Reverse primer:

5' AGGTTCTCATAA 3'

The multiplex primers for SNP TSC1104200 were:

Forward primer:

5' TGTCATCATTAA 3'

Reverse primer:

5' TGGTATTTGCAA 3'

The multiplex primers for SNP TSC0501389 were:

Forward primer:

5' TAGGGTTTGTAA 3'

Reverse primer:

5' CCCTAAGTAGAA 3'

The multiplex primers for SNP TSC0039960 were:

Forward primer:

5' GTATTTCTTTAA 3'

Reverse primer:

5' GAGTCTTCCCAA 3'

The multiplex primers for SNP TSC0418134 were:

Forward primer:

5' CAGGTAGAGTAA 3'

Reverse primer:

5' ATAGGATGTGAA 3'

The multiplex primers for SNP TSC0603688 were:

Forward primer:

5' CAATGTGTATAA 3'

Reverse primer:

5' AGAGGGCATCAA 3'

The multiplex primers for SNP TSC0129188 were:

Forward primer:

5' CCAGTGGTCTAA 3'

Reverse primer:

5' TAAACAATAGAA 3'

The multiplex primers for SNP TSC1103570 were:

Forward primer:

5' GCACACTTTTAA 3'

Reverse primer:

5' ATGGCTCTGCAA 3'

The multiplex primers for SNP TSC0813449 were:

Forward primer:

5' GTCATCTTGTAA 3'

Reverse primer:

5' TGCTTCATCTAA 3'

The multiplex primers for SNP TSC0701940 were:

Forward primer:

5' AGAAAGGGGCAA 3'

Reverse primer:

5' CTTTTCTTTCAA 3'

The multiplex primers for SNP TSC0087962 were:

Forward primer:

5' CTACTCTCTCAA 3'

Reverse primer:

5' ACAGCATTATAA 3'

The multiplex primers for SNP TSC0660274 were:

Forward primer:

5' ACTGCTCTGGAA 3'

Reverse primer:

5' GCAGAGGCACAA 3'

Multiplex PCR

Regions on chromosome 13 surrounding the above-mentioned 29 SNPs were amplified from the template genomic DNA using the polymerase chain reaction (PCR, U.S. Patent Nos. 4,683,195 and 4,683,202, incorporated herein by reference). This PCR reaction used primers that annealed approximately 150 bases upstream and downstream of each loci of interest. The fifty-eight primers were mixed together and used in a single reaction to amplify the template DNA. This reaction was done to increase the number of copies of the loci of interest, which eliminates error generated from a low number of genomes.

For increased specificity, a "hot-start" PCR reaction was used. PCR reactions were performed using the HotStarTaq Master Mix Kit supplied by QIAGEN (catalog number 203443). The amount of template DNA and primer per reaction can be optimized for each locus of interest. In this example, the 20 µl of plasma template DNA was used.

Two microliters of each forward and reverse primer, at concentrations of 5 mM were pooled into a single microcentrifuge tube and mixed. Four microliters of the primer mix was used in a total PCR reaction volume of 50 µl (20µl of template plasma DNA, 1

μl of sterile water, 4 μl of primer mix, and 25 μl of HotStar Taq. Twenty-five cycles of PCR were performed. The following PCR conditions were used:

(1) 95°C for 15 minutes;

(2) 95°C for 30 second;

(3) 4°C for 30 seconds;

(4) 37°C for 30 seconds;

(5) Repeat steps 2-4 twenty-four (24) times;

(6) 72°C for 10 minutes.

The temperatures and times for denaturing, annealing, and extension, can be optimized by trying various settings and using the parameters that yield the best results.

Other methods of genomic amplification can also be used to increase the copy number of the loci of interest including but not limited to primer extension preamplification (PEP) (Zhang *et al.*, PNAS, 89:5847-51, 1992), degenerate oligonucleotide primed PCR (DOP-PCR) (Telenius, *et al.*, Genomics 13:718-25, 1992), strand displacement amplification using DNA polymerase from bacteriophage 29, which undergoes rolling circle replication (Dean *et al.*, Genomic Research 11:1095-99, 2001), multiple displacement amplification (U.S. Patent 6,124,120), REPLI-g™ Whole Genome Amplification kits, and Tagged PCR.

Purification of Fragment of Interest

The unused primers, and nucleotides were removed from the reaction by using Qiagen MinElute PCR purification kits (Qiagen, Catalog Number 28004). The reactions were performed following the manufacturer's instructions supplied with the columns. The DNA was eluted in 100 μl of sterile water.

PCR Reaction Two

Design of Primers

SNPTSC0052277 was amplified using the following primer set:

First primer:

5' CTCCGTGGTATGGAATTCCACTCAAATCTTCATTCAGA 3'

Second primer:

5' ACGTCGGGTTACGGGACACCTGATTCCTC 3'

SNP TSC1225391 was amplified using the following primer set:

First primer:

5' TACCATTGGTTTGAATTCTTGTTTCCTGTTAACCATGC 3'

Second primer:

5' GCCGAGTTCTACGGGACAGAAAAGGGAGC 3'

SNP TSC0289078 was amplified using the following primer set:

First primer:

5' TGCAGTGATTTCGAATTCGAGACAATGCTGCCCAGTCA 3'

Second primer:

5' TCTAAATTCTCTGGGACCATTCCTTCAAC 3'

SNP TSC1349804 was amplified using the following primer set:

First primer:

5' ACTAACAGCACTGAATTCCATGCTCTTGGACTTTCCAT 3'

Second primer:

5' TCCCCTAACGTTGGGACACAGAATACTAC 3'

SNP TSC0870209 was amplified using the following primer set:

First primer:

5' GTCGACGATGGCGAATTCCTGCCACTCATTCAGTTAGC 3'

Second primer:

5' GAACGGCCCACAGGGACCTGGCATAACTC 3'

SNP TSC0194938 was amplified using the following primer set:

First primer:

5' TCATGGTAGCAGGAATTCTGCTTTGACCGATAAGGAGA 3'

Second primer:

5' ACTGTGGGATTCGGGACTGTCTACTACCC 3'

SNP TSC0820373 was amplified using the following primer set:

First primer:

5' ACCTCTCGGCCGGAATTCGGAAAAGTGTACAGATCATT 3'

Second primer:

5' GCCGGATACGAAGGGACGGCTCGTGACTC 3'

SNP TSC0902859 was amplified using the following primer set:

First primer:

5' CCGTAGACTAAAGAATTCCCTGATGTCAGGCTGTCACC 3'

Second primer:

5' ATCGGATCAGTCGGGACGGTGTCTTTGCC 3'

SNP TSC0501510 was amplified using the following primer set:

First primer:

5' GCATAGGCGGGAGAATTCCCTGTGTCCACCAAAGTCGG 3'

Second primer:

5' CCCACATAGGGCGGGACAAAGAGCTGAAC 3'

SNP TSC1228234 was amplified using the following primer set:

First primer:

5' GGCTTGCCGAGCGAATTCTAGGAAAGATACGGAATCAA 3'

Second primer:

5' TAACCCTCATACGGGACTTTCATGGAAGC 3'

SNP TSC0082910 was amplified using the following primer set:

First primer:

5' ATGAGCACCCGGGAATTCTGATTGGAGTCTAGGCCAAA 3'

Second primer:

5' TGCTCACCTTCTGGGACGTGGCTGGTCTC 3'

SNP TSC0838335 was amplified using the following primer set:

First primer:

5' ACCGTCTGCCACGAATTCTGGAAAACATGCAGTCTGGT 3'

Second primer:

5' TACACGGGAGGCGGGACAGGGTGATTAAC 3'

SNP TSC0818982 was amplified using the following primer set:

First primer:

5' CTTAAAGCTAACGAATTCAGAGCTGTATGAAGATGCTT 3'

Second primer:

5' AACGCTAAAGGGGGGACAACATAATTGGC 3'

SNP TSC0469204 was amplified using the following primer set:

First primer:

5' TTGTAAGAACGAGAATTCTGCAACCTGTCTTTATTGAA 3'

Second primer:

5' CTTCACCACTTTGGGACACTGAAGCCAAC 3'

SNP TSC1084457 was amplified using the following primer set:

First primer:

5' AACCATTGATTTGAATTCGAAATGTCCACCAAAGTTCA 3'

Second primer:

5' TGTCTAGTTCCAGGGACGCTGTTACTTAC 3'

SNP TSC0466177 was amplified using the following primer set:

First primer:

5' CGAAGGTAATGTGAATTCTGCCACAATTAAGACTTGGA 3'

Second primer:

5' ATACCGGTTTTCGGGACAGATCCATTGAC 3'

SNP TSC1270598 was amplified using the following primer set:

First primer:

5' CCTGAAATCCACGAATTCCACCCTGGCCTCCCAGTGCA 3'

Second primer:

5' TAGATGGTAGGTGGGACAGGACTGGCTTC 3'

SNP TSC1002017 was amplified using the following primer set:

First primer:

5' GCATATCTTAGCGAATTCCTGTGACTAATACAGAGTGC 3'

Second primer:

5' CCAAATATGGTAGGGACGTGTGAACACTC 3'

SNP TSC1104200 was amplified using the following primer set:

First primer:

5' TGCCGCTACAGGGAATTCATATGGCAGATATTCCTGAA 3'

Second primer:

5' ACGTTGCGGACCGGGACTTCCACAGAGCC 3'

SNP TSC0501389 was amplified using the following primer set:

First primer:

5' CTTCGCCCAATGGAATTCGGTACAGGGGTATGCCTTAT 3'

Second primer:

5' TGCACTTCTGCCGGGACCAGAGGAGAAAC 3'

SNP TSC0039960 was amplified using the following primer set:

First primer:

5' TGTGGGTATTCTGAATTCCACAAAATGGACTAACACGC 3'

Second primer:

5' ACGTCGTTCAGTGGGACATTAAAAGGCTC 3'

SNP TSC0418134 was amplified using the following primer set:

First primer:

5' GGTTATGTGTCAGAATTCTGAAACTAGTTTGGAAGTAC 3'

Second primer:

5' GCCTCAGTTTCGGGGACAGTTCTGAGGAC 3'

SNP TSC0603688 was amplified using the following primer set:

First primer:

5' TGTAACACGGCCGAATTCCTCATTTGTATGAAATAGGT 3'

Second primer:

5' AATCTAACTTGAGGGACCGGCACACACAC 3'

SNP TSC0129188 was amplified using the following primer set:

First primer:

5' AGTGTCCCCTTAGAATTCGCAGAGACACCACAGTGTGC 3'

Second primer:

5' TTTGCTACAGTCGGGACCCTTGTGTGCTC 3'

SNP TSC1103570 was amplified using the following primer set:

First primer:

5' AGCACATCACTAGAATTCAATACCATGTGTGAGCTCAA 3'

Second primer:

5' AATCCTGCTTCCGGGACCTAACTTTGAAC 3'

SNP TSC0813449 was amplified using the following primer set:

First primer:

5' TTTCATTTTCTGGAATTCCTCTAATGATTTTCTGGAGC 3'

Second primer:

5' CGTCGCCGCGTAGGGACTTTTTCTTCCAC 3'

SNP TSC0701940 was amplified using the following primer set:

First primer:

5' TTACTTAATCCTGAATTCGAGAAAAGCCATGTTGATAA 3'

Second primer:

5' TCATGGGTCGCTGGGACTTTGCCCTCTGC 3'

SNP TSC0087962 was amplified using the following primer set:

First primer:

5' ACTAACAGCACTGAATTCATTTTACTATAATCTGCTAC 3'

Second primer:

5' GTTAGCCGAGAAGGGACTGTCTGTGAAGC 3'

SNP TSC0660274 was amplified using the following primer set:

First primer:

5' AAATATGCAGCGGAATTCGTAAGTGACCTATTAATAAC 3'

Second primer:

5' GCGATGGTTACGGGGACAGCCAGGCAACC 3'

Each first primer had a biotin tag at the 5' end and contained a restriction enzyme recognition site for EcoRI, and was designed to anneal at a specified distance from the locus of interest. This allows a single reaction to be performed for the loci of interest, as each loci of interest will migrate at a distinct position (based on annealing position of first primer). The second primer contained a restriction enzyme recognition site for BsmF I.

All loci of interest were amplified from the multiplexed template DNA using the polymerase chain reaction (PCR, U.S. Patent Nos. 4,683,195 and 4,683,202, incorporated herein by reference). In this example, the loci of interest were amplified in separate reaction tubes but they could also be amplified together in a single PCR reaction. For

increased specificity, a "hot-start" PCR was used. PCR reactions were performed using the HotStarTaq Master Mix Kit supplied by QIAGEN (catalog number 203443).

The amount of multiplexed template DNA and primer per reaction can be optimized for each locus of interest. One microliter of the multiplexed template DNA eluted from the MinElute column was used in the PCR reaction for each locus of interest, and 5 μM of each primer was used. The twenty-nine SNPs described above also were amplified from the maternal DNA (15 ng of DNA was used in the PCR reaction; primer concentrations were as stated above). Forty cycles of PCR were performed. The following PCR conditions were used:

(1) 95°C for 15 minutes and 15 seconds;
(2) 37°C for 30 seconds;
(3) 95°C for 30 seconds;
(4) 57°C for 30 seconds;
(5) 95°C for 30 seconds;
(6) 64°C for 30 seconds;
(7) 95°C for 30 seconds;
(8) Repeat steps 6 and 7 thirty nine (39) times;
(9) 72°C for 5 minutes.

In the first cycle of PCR, the annealing temperature was about the melting temperature of the 3′ annealing region of the second primers, which was 37°C. The annealing temperature in the second cycle of PCR was about the melting temperature of the 3' region, which anneals to the template DNA, of the first primer, which was 57°C. The annealing temperature in the third cycle of PCR was about the melting temperature of the entire sequence of the second primer, which was 64°C. The annealing temperature for the remaining cycles was 64°C. Escalating the annealing temperature from TM1 to TM2 to TM3 in the first three cycles of PCR greatly improves specificity. These annealing temperatures are representative, and the skilled artisan will understand the annealing temperatures for each cycle are dependent on the specific primers used.

The temperatures and times for denaturing, annealing, and extension, can be optimized by trying various settings and using the parameters that yield the best results. In this example, the first primer was designed to anneal at various distances from the locus of interest. The skilled artisan understands that the annealing location of the first primer can be 5-10, 11-15, 16-20, 21-25, 26-30, 31-35, 36-40, 41-45, 46-50, 51-55, 56-

60, 61-65, 66-70, 71-75, 76-80, 81-85, 86-90, 91-95, 96-100, 101-105, 106-110, 111-115, 116-120, 121-125, 126-130, 131-140, 140-160, 160-180, 180-200, 200-220, 220-240, 240-260. 260-280. 280-300, 300-350, 350-400, 400-450, 450-500, or greater than 500 bases from the locus of interest.

Purification of Fragment of Interest

The PCR products were separated from the genomic template DNA. Each PCR product was placed into a well of a Streptawell, transparent, High-Bind plate from Roche Diagnostics GmbH (catalog number 1 645 692, as listed in Roche Molecular Biochemicals, 2001 Biochemicals Catalog). Alternatively, the PCR products can be pooled into a single well because the first primer was designed to allow the loci of interest to separate based on molecular weight. The first primers contained a 5' biotin tag so the PCR products bound to the Streptavidin coated wells while the genomic template DNA did not. The streptavidin binding reaction was performed using a Thermomixer (Eppendorf) at 1000 rpm for 20 min. at 37°C. Each well was aspirated to remove unbound material, and washed three times with 1X PBS, with gentle mixing (Kandpal et al., Nucl. Acids Res. 18:1789-1795 (1990); Kaneoka et al., Biotechniques 10:30-34 (1991); Green et al., Nucl. Acids Res. 18:6163-6164 (1990)).

Restriction Enzyme Digestion of Isolated Fragments

The purified PCR products were digested with the restriction enzyme BsmF I, which binds to the recognition site incorporated into the PCR products from the second primer. The digests were performed in the Streptawells following the instructions supplied with the restriction enzyme. After digestion, the wells were washed three times with PBS to remove the cleaved fragments.

Incorporation of Labeled Nucleotide

The restriction enzyme digest with BsmF I yielded a DNA fragment with a 5' overhang, which contained the SNP site or locus of interest and a 3' recessed end. The 5'

overhang functioned as a template allowing incorporation of a nucleotide or nucleotides in the presence of a DNA polymerase.

As demonstrated in Example 6, the sequence of both alleles of a SNP can be determined by filling in the overhang with one labeled nucleotide in the presence of the other unlabeled nucleotides. The following components were added to each fill in reaction: 1 µl of fluorescently labeled ddGTP, 0.5 µl of unlabeled ddNTPs (40 µM), which contained all nucleotides except guanine, 2 µl of 10X sequenase buffer, 0.25 µl of Sequenase, and water as needed for a 20µl reaction. The fill in reaction was performed at 40°C for 10 min. Non-fluorescently labeled ddNTP was purchased from Fermentas Inc. (Hanover, MD). All other labeling reagents were obtained from Amersham (Thermo Sequenase Dye Terminator Cycle Sequencing Core Kit, US 79565).

After labeling, each Streptawell was rinsed with 1X PBS (100 µl) three times. The "filled in" DNA fragments were then released from the Streptawells by digestion with the restriction enzyme EcoRI, according to the manufacturer's instructions that were supplied with the enzyme. Digestion was performed for 1 hour at 37 °C with shaking at 120 rpm.

Detection of the Locus of Interest

After release from the streptavidin matrix, the sample was loaded into a lane of a 36 cm 5% acrylamide (urea) gel (BioWhittaker Molecular Applications, Long Ranger Run Gel Packs, catalog number 50691). The sample was electrophoresed into the gel at 3000 volts for 3 min. The gel was run for 3 hours on a sequencing apparatus (Hoefer SQ3 Sequencer). The gel was removed from the apparatus and scanned on the Typhoon 9400 Variable Mode Imager. The incorporated labeled nucleotide was detected by fluorescence.

Below a schematic of the 5' overhang for SNP TSC0838335 is depicted. The entire sequence is not reproduced, only a portion to depict the overhang (where R indicates the variable site).

10/14	5' TAA				
	3' ATT	R	A	C	A
Overhang position		1	2	3	4

The observed nucleotides for TSC0838335 are adenine and guanine on the 5' sense strand (herein depicted as the top strand). The nucleotide in position three of the overhang corresponded to cytosine, which is complementary to guanine. Labeled ddGTP can be used to determine the sequence of both allele in the presence of unlabeled dATP, dCTP, and dTTP.

The restriction enzyme BsmF I was used to create the 5' overhang, which typically cuts 10/14 from the recognition site. At times, BsmF I will cut 11/15 from the recognition site and generate the following overhang:

11/15	5' TA				
	3' AT	T	R	A	C
Overhang position		0	1	2	3

Position 0 in the overhang is thymidine, which is complementary to adenine. Position 0 complementary to the overhang was filled in with unlabeled dATP, and thus after the fill-in reaction, the exact same molecules were generated whether the enzyme cut at 10/14 or 11/15 from the recognition site. The DNA molecules generated after the fill-in reaction are depicted below:

G allele 10/14	5' TAA	**G***			
	3' ATT	C	A	C	A
Overhang position		**1**	2	3	4
G allele 11/15	5' TA	A	**G***		
	3' AT	T	C	A	C
Overhang position		0	**1**	2	3
A allele 10/14	5' TAA A	T	**G***		
	3' ATT T	A	C	A	
Overhang position		1	2	**3**	4
A allele 11/15	5' TA	A	A	T	**G***

	3' AT	T	T	A	C
Overhang position		0	1	2	3

The maternal template DNA amplified for TSC0838335 displayed a single band that migrated at the expected position of the higher molecular weight band, which corresponded to the "A" allele (see FIG. 20, lane 1). The maternal template DNA was homozygous for adenine at SNP TSC0838335.

However, in lane 2, amplification of the multiplexed template DNA for TSC0838335 isolated from the plasma of the same individual displayed two bands; a lower molecular weight band, which corresponded to the "G" allele, and the higher molecular weight band, which corresponded to the "A" allele. The template DNA isolated from the plasma of a pregnant female contains both maternal template DNA and fetal template DNA.

As seen in FIG. 20, lane 1, the maternal template DNA was homozygous for adenine at this SNP (compare lanes 1 and 2). The "G" allele represented the fetal DNA. Signals from the maternal template DNA and the fetal template DNA clearly have been distinguished. The "G" allele becomes a beacon for the fetal DNA and can be used to measure the amount of fetal DNA present in the sample. Additionally, once the percentage of fetal DNA in the maternal plasma for a given sample has been determined, any deviation from this percentage indicates a chromosomal abnormality. This method provides the first non-invasive method for the detection of fetal chromosomal abnormalities.

As seen in FIG. 20, lane 3, analysis of the maternal DNA for SNP TSC0418134 generated a single band that migrated at the expected position of the higher molecular weight band, which corresponded to the adenine allele. Likewise, analysis of the multiplexed template DNA isolated from the maternal plasma gave a single band, which migrated at the expected position of the adenine allele (see FIG. 20, lane 4). Both the maternal DNA and the fetal DNA are homozygous for adenine at TSC0418134.

Below, a schematic of the 5' overhang for TSC0129188 is depicted, wherein R indicates the variable site:

10/14	5' TCAT				
	3' AGTA	R	A	C	T

Overhang position	1	2	3	4	

The nucleotide upstream of the variable site (R) does not correspond to guanine on the sense strand. Thus, the 5' overhang generated by the 11/15 cutting properties of BsmF I will be filled-in identically to the 5'overhang generated by the 10/14 cut. Labeled ddGTP in the presence of unlabeled dATP, dTTP, and dCTP was used for the fill-in reaction. The DNA molecules generated after the fill-in reaction are depicted below:

A allele 10/14	5' TCAT	**A**	T	**G***	
	3' AGTA	T	A	C	T
Overhang position		1	2	3	4
G allele 10/14	5' TCAT	**G***			
	3' AGTA	C	A	C	T
Overhang position		1	2	3	4

Analysis of the maternal DNA for SNP TSC0129188 gave a single band that corresponded to the DNA molecules filled in with ddGTP at position 1 complementary to the overhang, which represented the "G" allele (see FIG. 20, lane 5). No band was detected for adenine allele, indicating that the maternal DNA is homozygous for guanine.

In contrast, analysis of the multiplexed template DNA from the maternal plasma, which contains both maternal DNA, and fetal DNA, gave two distinct bands (see FIG. 20, lane 6). The lower molecular weight band corresponded to the "G" allele, while the higher molecular weight corresponded to the "A" allele. The "A" allele represents the fetal DNA. Thus, a method has been developed that allows separation of maternal DNA and fetal DNA signals without the added complexity of having to isolate fetal cells. In addition, a sample of paternal DNA is not required to detect differences between the maternal DNA and the fetal DNA.

Analysis of the maternal DNA for SNP TSC0501389 gave a single band that migrated at the higher molecular weight position, which corresponded to the "A" allele. No band was detected that corresponded to the "G" allele. Similarly, analysis of the multiplexed template DNA from the maternal plasma for SNP TSC0501389 gave a single band that migrated at the higher molecular weight position, which corresponded to the

"A" allele. Both the maternal template DNA and the fetal template DNA were homozygous for adenine at SNP TSC0501389.

The maternal DNA and the template DNA from the plasma originated from the same sample. One sample, which was obtained through a non-invasive procedure, provided a genetic fingerprint for both the mother and the fetus.

Of the twenty-nine SNPs for which the maternal template DNA was homozygous, the fetal template DNA was heterozygous at two of the twenty-nine SNPs. The fetal DNA was homozygous for the same allele as the maternal template DNA at the remaining 27 SNPs (data not shown). Comparing the homozygous allele of the maternal template DNA and the plasma template DNA at a given SNP provides an added level of quality control. It is not possible that the maternal template DNA and the plasma template DNA are homozygous for different alleles at the same SNP. If this is seen, it would indicate that an error in processing had occurred.

The methods described herein demonstrate that the maternal genetic signal can be separated and distinguished from the fetal genetic signal in a maternal plasma sample. The above-example analyzed SNPs located on chromosome 13, however any chromosome can be analyzed including human chromosome 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X and Y and fetal chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X and Y.

In addition, the methods described herein can be used to detect fetal DNA in any biological sample including but not limited to cell, tissue, blood, serum, plasma, saliva, urine, tears, vaginal secretions, umbilical cord blood, chorionic villi, amniotic fluid, embryonic tissues, lymph fluid, cerebrospinal fluid, mucosa secretions, peritoneal fluid, ascitic fluid, fecal matter, or body exudates.

The methods described herein demonstrate that the percentage of fetal DNA in the maternal sample can be determined by analyzing SNPs wherein the maternal DNA is homozygous, and the DNA isolated from the plasma of the pregnant female is heterozygous. The percentage of fetal DNA can be used to determine if the fetal genotype has any chromosomal disorders.

For example, if the percentage of fetal DNA present in the sample is calculated to be 30% by analysis of chromosome 1 (chromosomal abnormalities involving chromosome 1 terminate early in the pregnancy), then any deviation from 30% fetal DNA is indicative of a chromosomal abnormality. For example, if upon analysis of a SNP or

multiple SNPs on chromosome 18, the percentage of fetal DNA is higher than 30%, this would indicate that an additional copy of chromosome 18 is present. The calculated percentage of fetal DNA from any chromosome can be compared to any other chromosome. In particular, the percentage of fetal DNA on chromosome 13 can be compared to the percentage of fetal DNA on chromosomes 18 and 21.

This analysis is assisted by knowledge of the expected ratio of one allele to the other allele at each SNP. As discussed in Example 9, not all heterozygous SNPs display ratios of 50:50. Knowledge of the expected ratio of one allele to the other reduces the overall number of variable sites that must be analyzed. However, even without knowledge of the expected ratios for the various SNPs, the percentage of fetal DNA can be calculated by analyzing a large number of SNPs. When the sampling size of SNPs is large enough, the statistical variation arising from the values of the expected ratios will be eliminated.

In addition, heterozygous maternal SNPs also provide valuable information. The analysis is not limited to homozygous maternal SNPs. For example, if at a heterozygous SNP on maternal DNA, the ratio of allele I to allele 2 is 1:1, then in the plasma template DNA the ratio should remain 1:1 unless the fetal DNA carries a chromosomal abnormality.

The above methods can also be used to detect mutations in the fetal DNA including but not limited to point mutations, transitions, transversions, translocations, insertions, deletions, and duplications. As seen in FIG. 20, fetal DNA can readily be distinguished from maternal DNA. The above methods can be used to determine the sequence of any locus of interest for any gene.

Having now fully described the invention, it will be understood by those of skill in the art that the invention can be performed with a wide and equivalent range of conditions, parameters, and the like, without affecting the spirit or scope of the invention or any embodiment thereof.

All documents, e.g., scientific publications, patents and patent publications recited herein are hereby incorporated by reference in their entirety to the same extent as if each individual document was specifically and individually indicated to be incorporated by reference in its entirety. Where the document cited only provides the first page of the document, the entire document is intended, including the remaining pages of the document.

WHAT IS CLAIMED IS:

1. A method for detecting a chromosomal abnormality, said method comprising:

(a) determining the sequence of alleles of a locus of interest from template DNA,

(b) quantitating the relative amount of the alleles at a heterozygous locus of interest that was identified from the locus of interest of (a), wherein said relative amount is expressed as a ratio, and wherein said ratio indicates the presence or absence of a chromosomal abnormality.

2. The method of claim 1, wherein said template DNA is obtained from a source selected from the group consisting of human, non-human, mammal, reptile, cattle, cat, dog, goat, swine, pig, monkey, ape, gorilla, bull, cow, bear, horse, sheep, poultry, mouse, rat, fish, dolphin, whale, and shark.

3. The method of claim 2, wherein the template DNA is obtained from a human source.

4. The method of claim 1, wherein the template DNA is obtained from a sample selected from the group consisting of: a cell, fetal cell, tissue, blood, serum, plasma, saliva, urine, tear, vaginal secretion, umbilical cord blood, chorionic villi, amniotic fluid, embryonic tissue, an embryo, a four-celled embryo, an eight celled embryo, a 16-celled embryo, a 32-celled embryo, a 64-celled embryo, a 128-celled embryo, a 256-celled embryo, a 512-celled embryo, a 1024-celled embryo, lymph fluid, cerebrospinal fluid, mucosa secretion, peritoneal fluid, ascitic fluid, fecal matter, or body exudates.

5. The method of claim 1, wherein alleles of multiple loci of interest are sequenced and their relative amounts quantitated and expressed as a ratio.

6. The method of claim 5, wherein said multiple loci of interest are on multiple chromosomes.

7. The method of claim 3, wherein said human is a pregnant female.

8. The method of claim 7, wherein template DNA from said pregnant female is obtained from a sample selected from the group consisting of: cells, tissues, blood, serum, plasma, saliva, urine, tear, vaginal secretion, lymph fluid, cerebrospinal fluid, mucosa secretion, peritoneal fluid, ascitic fluid, fecal matter, umbilical cord blood, chorionic villi, amniotic fluid and body exudate.

9. The method of claim 4, wherein said sample is mixed with a cell lysis inhibitor.

10. The method of claim 9, wherein said cell lysis inhibitor is selected from the group consisting of glutaraldehyde, derivatives of glutaraldehyde, formaldehyde, formalin, and derivatives of formaldehyde.

11. The method of claim 9, wherein said sample is blood.

12. The method of claim 9, wherein said sample is blood from a pregnant female.

13. The method of claim 12, wherein said blood is obtained from a human pregnant female when the fetus is at a gestational age selected from the group consisting of: 0-4, 4-8, 8-12, 12-16, 16-20, 20-24, 24-28, 28-32, 32-36, 36-40, 40-44, 44-48, 48-52, and more than 52 weeks.

14. The method of claim 12, wherein said template DNA is obtained from plasma from said blood.

15. The method of claim 12, wherein said template DNA is obtained from serum from said blood.

16. The method of claim 14 or claim 15, wherein said template DNA comprises a mixture of maternal DNA and fetal DNA.

17. The method of claim 16, wherein prior to (a), maternal DNA is sequenced to identify a homozygous locus of interest, and further wherein said homozygous locus of interest is the locus of interest analyzed in the template DNA of (a).

18. The method of claim 16, wherein prior to (a), maternal DNA is sequenced to identify a heterozygous locus of interest, and further wherein said heterozygous locus of interest is the locus of interest analyzed in the template DNA of (a).

19. The method of claim 1, wherein determining the sequence of the alleles comprises:

(a) amplifying alleles of a locus of interest on a template DNA using a first and a second primer, wherein the second primer contains a recognition site for a restriction enzyme such that digestion with the restriction enzyme generates a 5' overhang containing the locus of interest;

(b) digesting the amplified DNA with the restriction enzyme that recognizes the recognition site on the second primer;

(c) incorporating a nucleotide into the digested DNA of (b) by using the 5' overhang containing the locus of interest as a template; and

(d) determining the sequence of the alleles of the locus of interest by determining the sequence of the DNA of (c).

20. The method of claim 19, wherein said first and second primers contain a portion of a restriction enzyme recognition site that contains a variable nucleotide, wherein the full restriction enzyme recognition site is generated after amplification.

21. The method of claim 20, wherein the restriction enzyme recognition site is for a restriction enzyme selected from the group consisting of BsaJ I, Bssk I, Dde I, EcoN I, Fnu4H I, Hinf I, and ScrF I.

22. The method of claim 19, wherein the restriction enzyme cuts DNA at a distance from the recognition site.

23. The method of claim 22, wherein the recognition site is for a Type IIS restriction enzyme.

24. The method of claim 23, wherein the Type IIS restriction enzyme is selected from the group consisting of: Alw I, Alw26 I, Bbs I, Bbv I, BceA I, Bmr I, Bsa I, Bst71 I, BsmA I, BsmB I, BsmF I, BspM I, Ear I, Fau I, Fok I, Hga I, Ple I, Sap I, SSfaN I, and Sthi32 I.

25. The method of claim 19, wherein said method of amplification is selected from the group consisting of: polymerase chain reaction, self-sustained sequence reaction, ligase chain reaction, rapid amplification of cDNA ends, polymerase chain reaction and ligase chain reaction, Q-beta phage amplification, strand displacement amplification, and splice overlap extension polymerase chain reaction.

26. The method of claim 25, wherein said method of amplification is PCR.

27. The method of claim 26, wherein an annealing temperature for cycle 1 of PCR is about the melting temperature of the portion of the 3' region of the second primer that anneals to the template DNA.

28. The method of claim 27, wherein an annealing temperature for cycle 2 of PCR is about the melting temperature of the portion of the 3' region of the first primer that anneals to the template DNA.

29. The method of claim 28, wherein an annealing temperature for the remaining cycles of PCR is at about the melting temperature of the entire second primer.

30. The method of claim 1, wherein determining the sequence comprises a method selected from the group consisting of: allele specific PCR, mass spectrometry, hybridization, primer extension, fluorescence resonance energy transfer (FRET), sequencing, Sanger dideoxy sequencing, DNA micorarray, southern blot, slot blot, dot blot, and MALDI-TOF mass spectrometry.

31. The method of claim 1, wherein said ratio for alleles at heterozygous loci of interest on a chromosome are summed and compared to the ratio for alleles at heterozygous loci of interest on a different chromosome, wherein a difference in ratios indicates the presence of a chromosomal abnormality.

32. The method of claim 31, wherein the chromosomes that are compared are human chromosomes selected from the group consisting of: chromosome 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X, and Y.

33. The method of claim 31, wherein the ratio for the alleles at heterozygous loci of interest of chromosomes 13, 18, and 21 are compared.

34. The method of claim 1, wherein said locus of interest is a single nucleotide polymorphism.

35. The method of claim 1, wherein said locus of interest is a mutation.

36. A method for determining the sequence of a locus of interest on fetal DNA, said method comprising:

(a) obtaining template DNA from a sample from a pregnant female, wherein said template DNA comprises fetal DNA and maternal DNA;

(b) adding a cell lysis inhibitor to said sample of (a); and

(c) determining the sequence of a locus of interest on template DNA from said sample of (b).

37. The method of claim 36, wherein said sample from pregnant female is selected from the group consisting of: tissue, cell, blood, serum, plasma, urine, and vaginal secretion.

38. The method of claim 37, wherein said sample is blood.

39. The method of claim 36, wherein said cell lysis inhibitor is selected from the group consisting of: glutaraldehyde, derivatives of glutaraldehyde, formaldehyde, derivatives of formaldehyde, and formalin.

40. The method of claim 36, wherein prior to step (c), template DNA is isolated.

41. The method of claim 38, wherein said template DNA is obtained from plasma of said blood.

42. The method of claim 38, wherein said template DNA is obtained from serum of said blood.

43. The method of claim 36, wherein prior to step (c), the sequence of the locus of interest on maternal template DNA is determined.

44. The method of claim 36, wherein prior to step (c), the sequence of the locus of interest on paternal template DNA is determined.

45. The method of claim 36, wherein said locus of interest is a single nucleotide polymorphism.

46. The method of claim 36, wherein said locus of interest is a mutation.

47. The method of claim 36, wherein the sequence of multiple loci of interest is determined.

48. The method of claim 47, wherein the multiple loci of interest are on multiple chromosomes.

49. The method of claim 36, wherein determining the sequence comprises:

(a) amplifying a locus of interest on a template DNA using a first and second primers, wherein the second primer contains a recognition site for a restriction

enzyme such that digestion with the restriction enzyme generates a 5' overhang containing the locus of interest;

(b) digesting the amplified DNA with the restriction enzyme that recognizes the recognition site on the second primer;

(c) incorporating a nucleotide into the digested DNA of (b) by using the 5' overhang containing the locus of interest as a template; and

(d) determining the sequence of the locus of interest by determining the sequence of the DNA of (c).

50. The method of claim 49, wherein said first and second primers contain a portion of a restriction enzyme recognition site that contains a variable nucleotide, wherein the full restriction enzyme recognition site is generated after amplification.

51. The method of claim 50, wherein the restriction enzyme is selected from the group consisting of BsaJ I, Bssk I, Dde I, EcoN I, Fnu4H I, Hinf I and ScrF I.

52. The method of claim 49, wherein the restriction enzyme cuts DNA at a distance from the recognition site.

53. The method of claim 52, wherein the recognition site is for a Type IIS restriction enzyme.

54. The method of claim 53, wherein the Type IIS restriction enzyme is selected from the group consisting of: Alw I, Alw26 I, Bbs I, Bbv I, BceA I, Bmr I, Bsa I, Bst71 I, BsmA I, BsmB I, BsmF I, BspM I, Ear I, Fau I, Fok I, Hga I, Ple I, Sap I, SSfaN I, and Sthi32 I.

55. The method of claim 49, wherein said method of amplification is selected from the group consisting of: polymerase chain reaction, self-sustained sequence reaction, ligase chain reaction, rapid amplification of cDNA ends, polymerase chain reaction and ligase chain reaction, Q-beta phage amplification, strand displacement amplification, and splice overlap extension polymerase chain reaction.

56. The method of claim 55, wherein said method of amplification is by PCR.

57. The method of claim 56, wherein an annealing temperature for cycle 1 of PCR is about the melting temperature of the portion of the 3' region of the second primer that anneals to the template DNA.

58. The method of claim 57, wherein an annealing temperature for cycle 2 of PCR is about the melting temperature of the portion of the 3' region of the first primer that anneals to the template DNA.

59. The method of claim 58, wherein an annealing temperature for the remaining cycles of PCR is at about the melting temperature of the entire second primer.

60. The method of claim 36, wherein the sequence of a locus of interest is determined using a method selected from the group consisting of: allele specific PCR, mass spectrometry, hybridization, primer extension, fluorescence polarization, fluorescence resonance energy transfer (FRET), fluorescence detection, sequencing, Sanger dideoxy sequencing, DNA micorarray, southern blot, slot blot, dot blot, and MALDI-TOF mass spectrometry.

61. A method for determining the sequence of a locus of interest on fetal DNA, said method comprising:

(a) amplifying a locus of interest on a template DNA using a first and second primers, wherein the second primer contains a recognition site for a restriction enzyme such that digestion with the restriction enzyme generates a 5' overhang containing the locus of interest;

(b) digesting the amplified DNA with the. restriction enzyme that recognizes the recognition site on the second primer;

(c) incorporating a nucleotide into the digested DNA of (b) by using the 5' overhang containing the locus of interest as a template; and

(d) determining the sequence of the locus of interest by determining the sequence of the DNA of (c).

62. The method of claim 61, further comprising obtaining template DNA from a sample from a pregnant female, wherein said template DNA comprises fetal DNA and maternal DNA and adding a cell lysis inhibitor to the sample from the pregnant female.

63. The method of claim 62, wherein said sample from pregnant female is selected from the group consisting of: tissue, cell, blood, serum, plasma, urine, and vaginal secretion.

64. The method of claim 63, wherein said sample is blood.

65. The method of claim 62, wherein said cell lysis inhibitor is selected from the group consisting of: glutaraldehyde, derivatives of glutaraldehyde, formaldehyde, derivatives of formaldehyde, and formalin.

66. A kit for use in any of the methods of claims 1 to 65 comprising a set of primers used in the method, wherein the second primer contains a sequence that generates a recognition site for a restriction enzyme such that digestion with the restriction enzyme generates a 5"overhang containing the locus of interest, and a set of instructions.

FIG. 1A

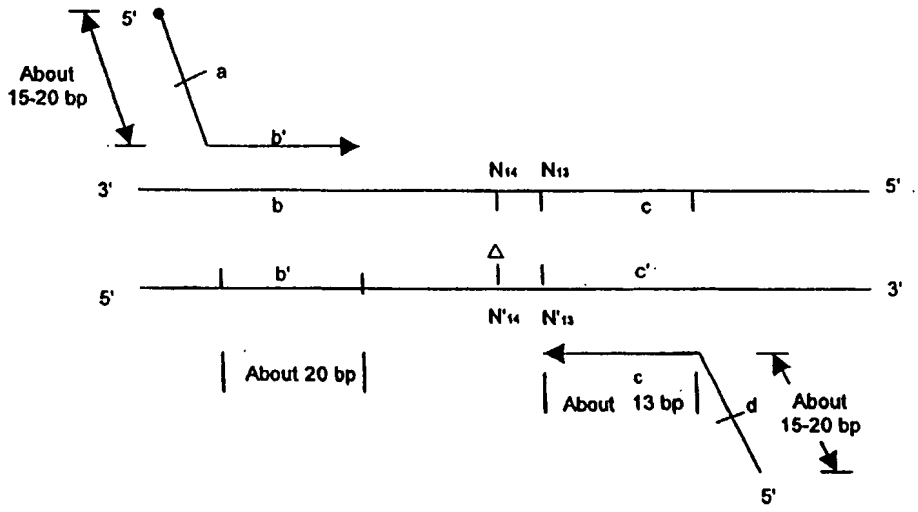

5'
About
15-20 bp
a
b'
3'
N14 N13
5'
b
c
b'
c'
5'
3'
N'14 N'13
About 20 bp
c
About 13 bp
d
About
15-20 bp
5'

FIG. 1B

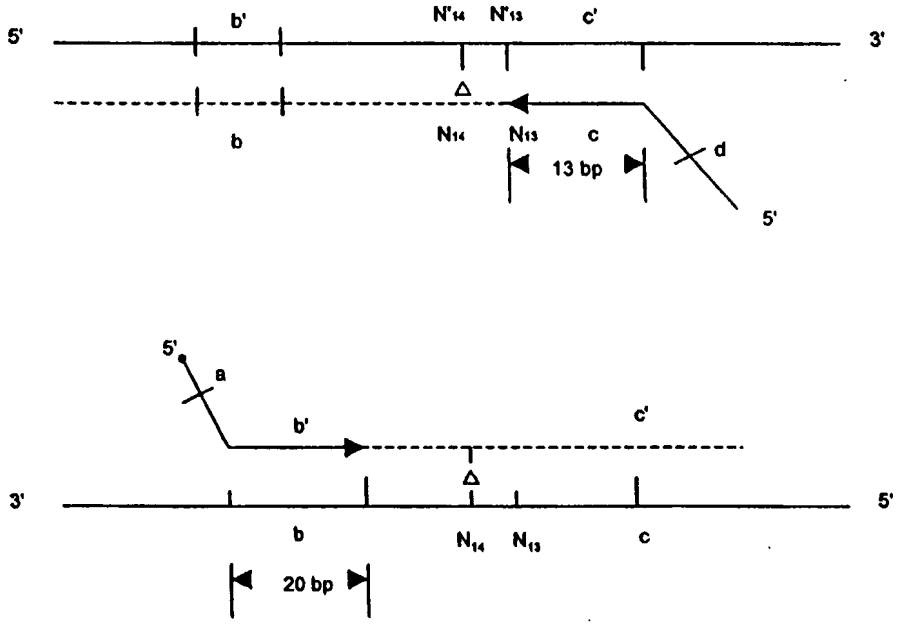


FIG. 1C

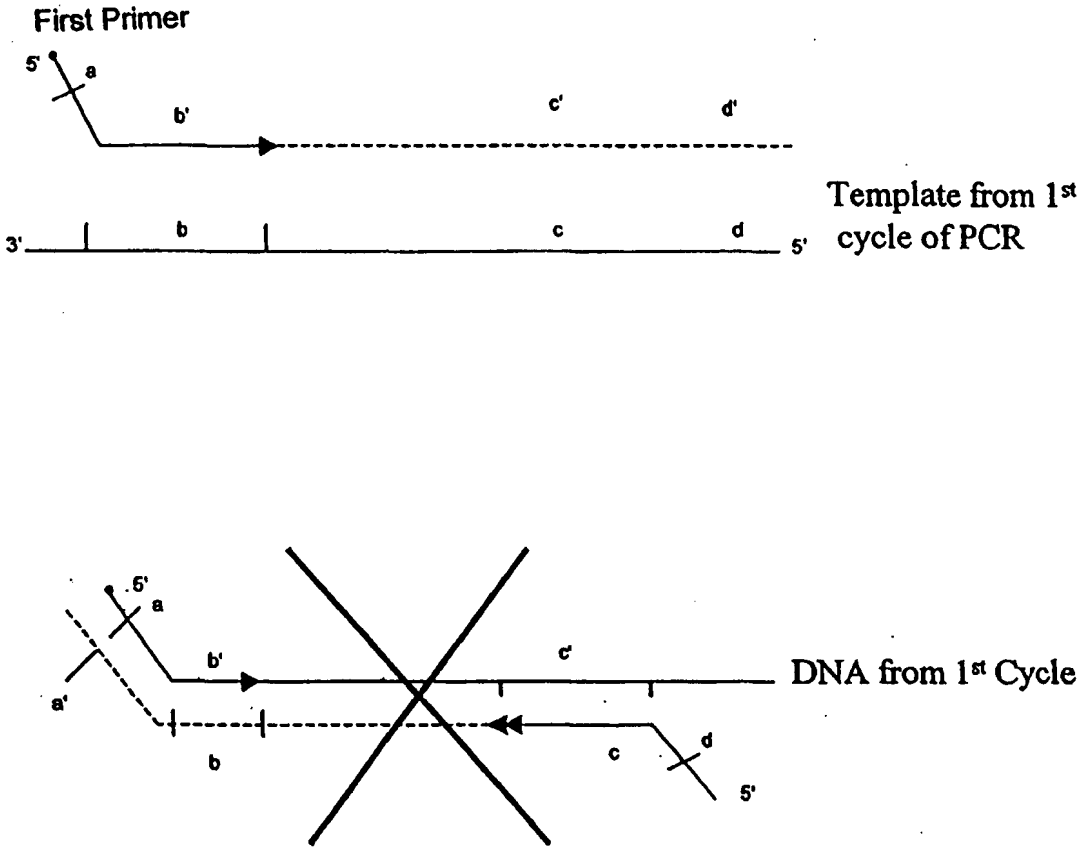

First Primer
5'
a
b'
c'
d'
Template from 1st cycle of PCR
3'
b
c
d
5'
5'
a
b'
c'
DNA from 1st Cycle
a'
b
c
d
5'

FIG. 1D

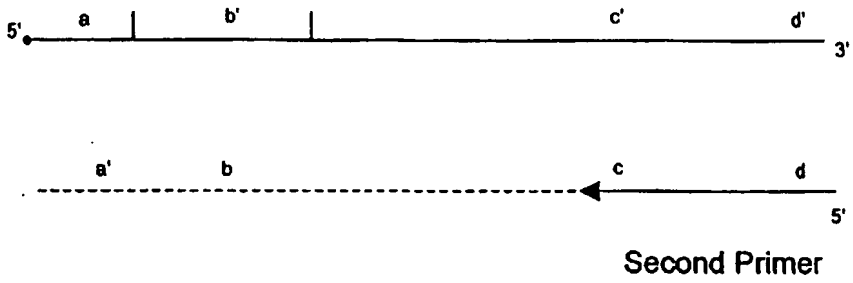

5'
a
b'
c'
d'
3'
Template from 2nd cycle of PCR
a'
b
c
d
5'
Second Primer

FIG. 1E

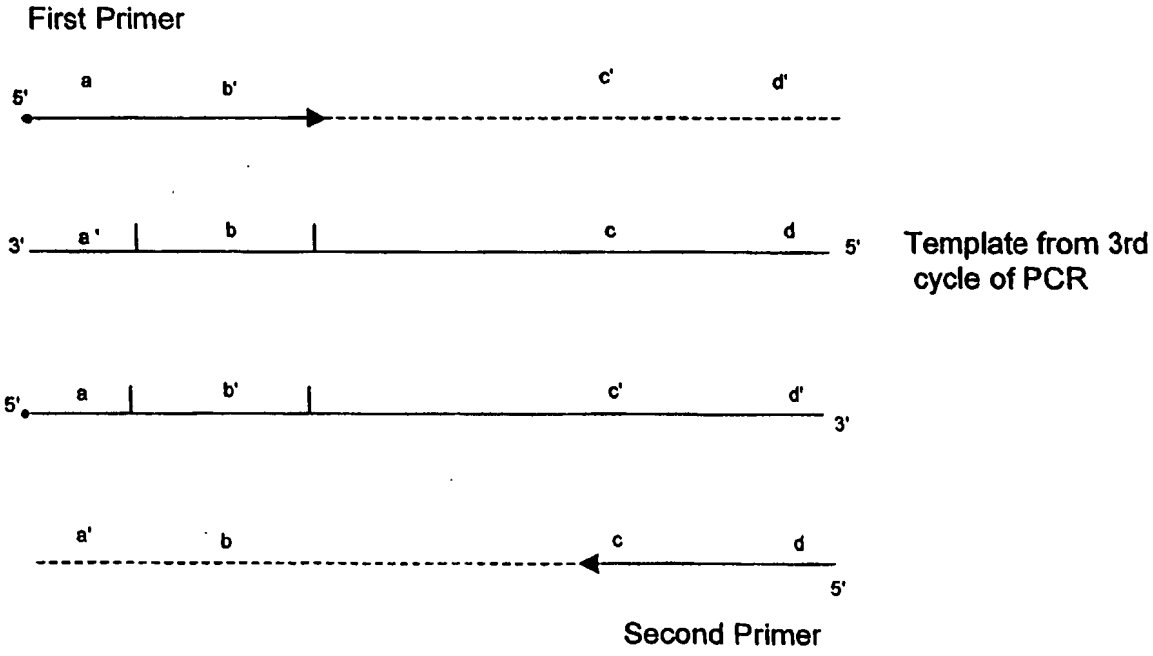

First Primer
5'
a
b'
c'
d'
3'
a'
b
c
d
5'
Template from 3rd cycle of PCR
5'
a
b'
c'
d'
3'
a'
b
c
d
5'
Second Primer

FIG. 1F

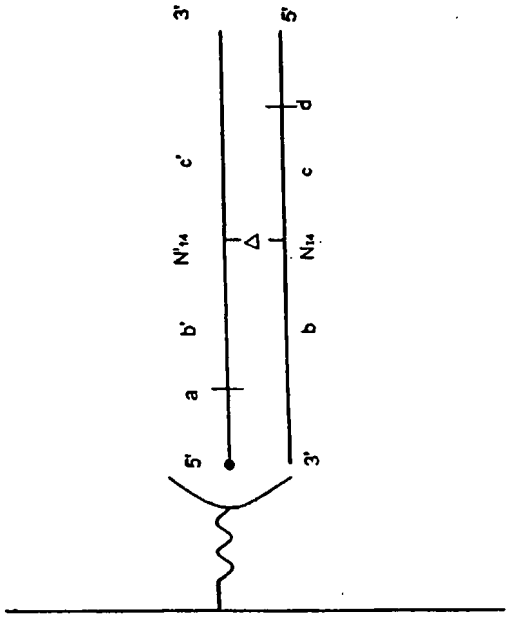


FIG. 1G

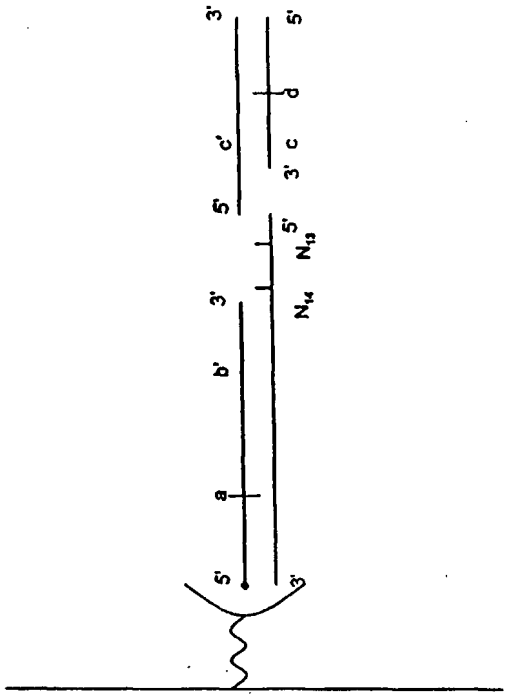


FIG. 1H

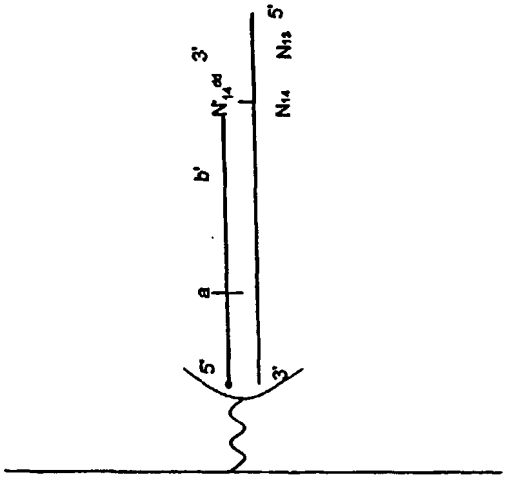


FIG. 1I

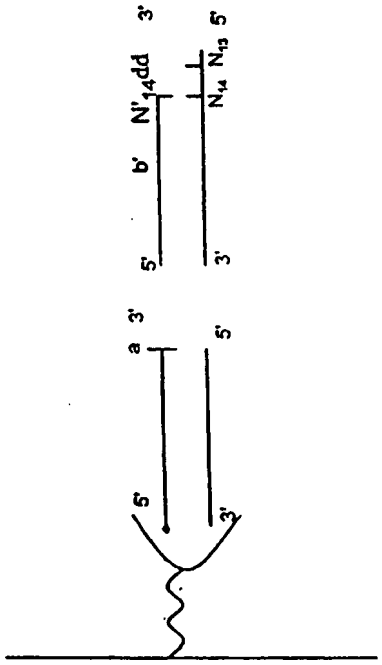


FIG. 2

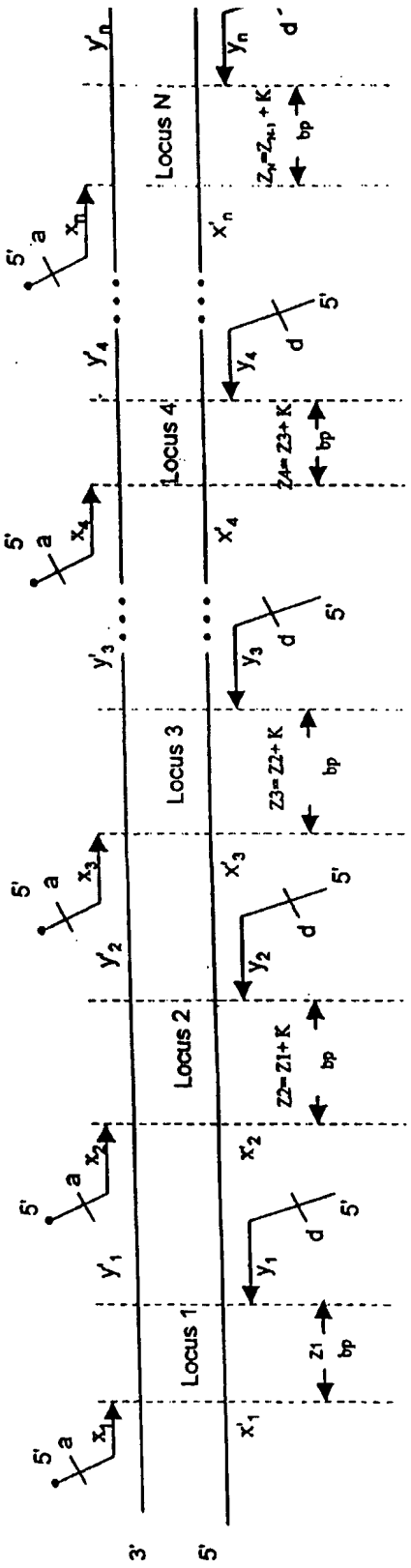

Locus N
Locus 4
Locus 3
Locus 2
Locus 1
3'
5'
5'
a
d
bp
Z1
Z2= Z1+ K
Z3= Z2+ K
Z4= Z3+ K
Zn=Zn-1 + K

FIG. 4A

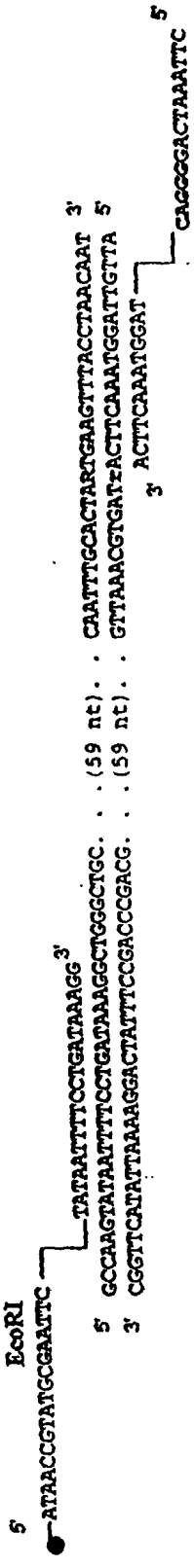
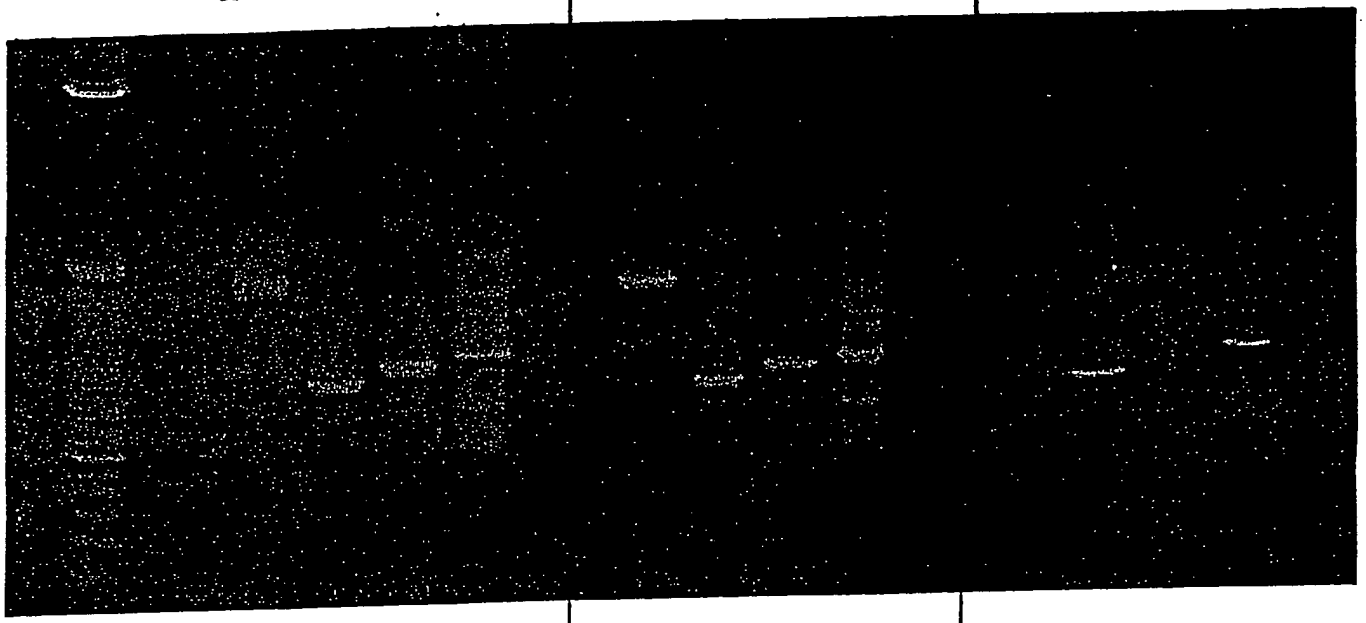

5'
EcoRI
ATAACCGTATGCGAATTC
TATAATTTTCCTGATAAAGG 3'
5' GCCAAGTATAATTTTCCTGATAAAGGCTGGGCTGC. . .(59 nt). . CAATTGCACTATGAAGTTTACCTAACAAT 3'
3' CGGTTCATATTAAAAGGACTATTTCCGACCCGACG. . .(59 nt). . GTTAAACGTGATACTTCAAATGGATTGTTA 5'
3' ACTTCAAATGGAT
CAGGGACTAAATTC 5'
BsmFI

FIG. 4B

5'
EcoRI
ATAACCGTATGCGAATTC
TATAATTTTCCTGATAAAGG 3'
5' GCCAAGTATAATTTTCCTGATAAAGGCTGGGCTGC. . .(59 nt). . CAATTGCACTATGAAGTTTACCTAACAAT 3'
3' CGGTTCATATTAAAAGGACTATTTCCGACCCGACG. . .(59 nt). . GTTAAACGTGATACTTCAAATGGATTGTTA 5'
3' ACTTCAAATGGAT
CGGCAGACTAAATTC
BceAI

FIG. 3A FIG. 3B FIG. 3C



Lane	M	1	2	3	4	1	2	3	4	1	2	3	4
Annealing Temp. 1st Cycle (TM1)		37°C				40°C				46°C			
Annealing Temp. 2nd Cycle (TM2)		57°C				60°C				65°C			
Annealing Temp. 3rd Cycle (TM3)		64°C				67°C				72°C			

FIG. 4C

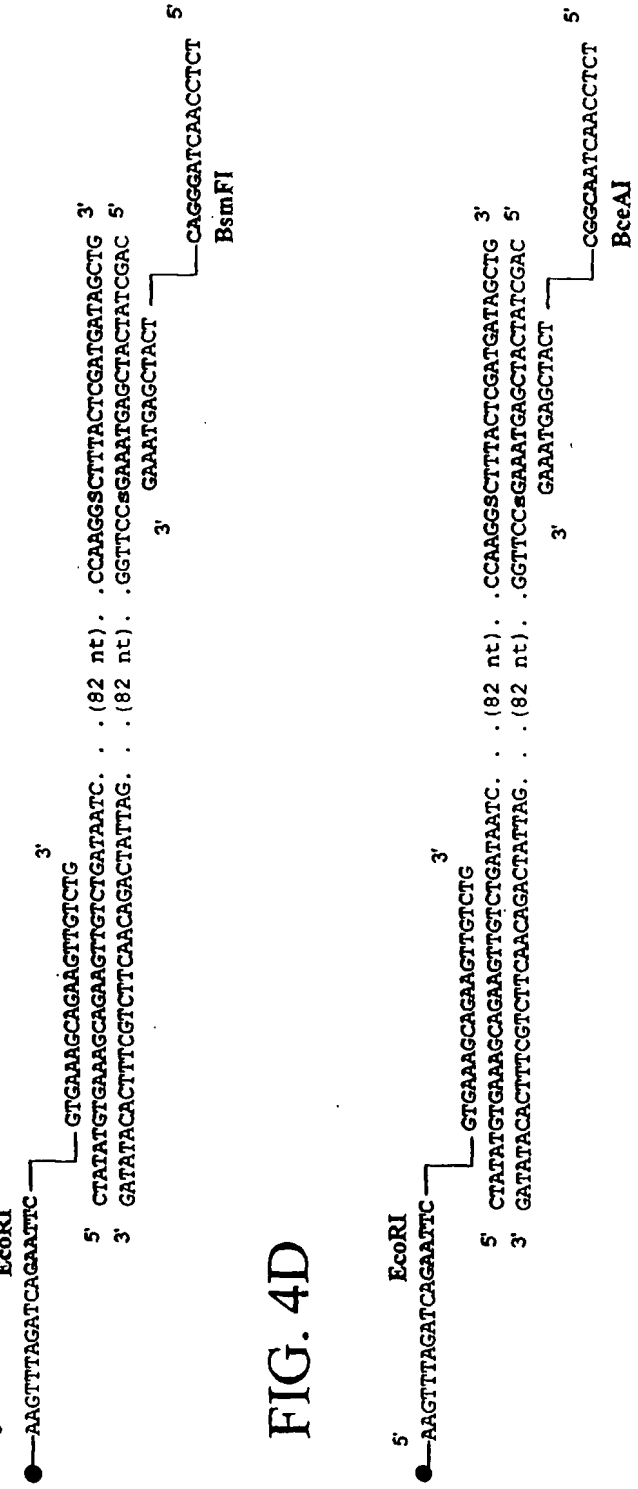

5'
EcoRI
AAGTTTAGATCAGAATTC
3'
GTGAAAGCAGAAGTTGTCTG
5' CTATATGTGAAAGCAGAAGTTGTCTGATAATC. . .(82 nt). .CCAAGGSCTTTACTCGATGATAGCTG 3'
3' GATATACACTTTCGTCTTCAACAGACTATTAG. . .(82 nt). .GGTTCCsGAAATGAGCTACTATCGAC 5'
3' GAAATGAGCTACT
5'
CAGGGATCAACCTCT
BsmFI

FIG. 4D

5'
EcoRI
AAGTTTAGATCAGAATTC
3'
GTGAAAGCAGAAGTTGTCTG
5' CTATATGTGAAAGCAGAAGTTGTCTGATAATC. . .(82 nt). .CCAAGGSCTTTACTCGATGATAGCTG 3'
3' GATATACACTTTCGTCTTCAACAGACTATTAG. . .(82 nt). .GGTTCCsGAAATGAGCTACTATCGAC 5'
3' GAAATGAGCTACT
5'
CGGCAATCAACCTCT
BceAI

FIG. 5A


EcoRI
5'
ATAACCGTATGCGAATTCTATAATTTTCCTGATAAAGGCTG. . .(59 nt). . CAATTTGCACTARTGAAGTTTACCTAGTCCCCAGATTTAAG 3'
3' TATTGGCATACGCTTAAGATATTAAAAGGACTATTTCCGAC. . .(59 nt). . GTTAAACGTGATCAGGGACTAAATTC 5'
BsmFI

FIG. 5B

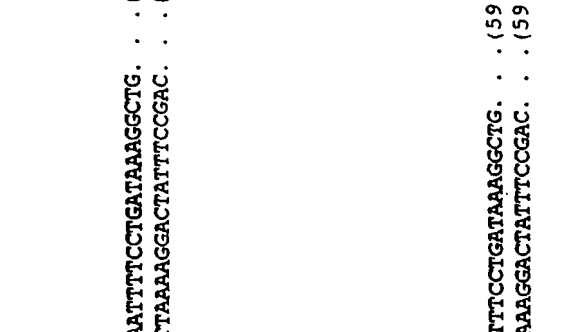

EcoRI
3'
5'
ATAACCGTATGCGAATTCTATAATTTTCCTGATAAAGGCTG. . .(59 nt). . CAATTTGCACTARTGAAGTTTACCTAGCCGTCAGATTTAAG
3' TATTGGCATACGCTTAAGATATTAAAAGGACTATTTCCGAC. . .(59 nt). . GTTAAACGTGATCAGGCAGACTAAATTC 5'
BceAI

FIG. 5C

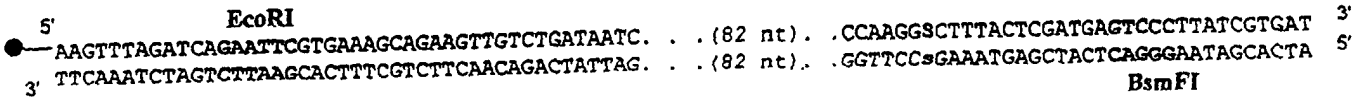

5'
EcoRI
AAGTTTAGATCAGAATTCGTGAAAGCAGAAGTTGTCTGATAATC. . .(82 nt). .CCAAGGSCTTTACTCGATGAGTCCCTTATCGTGAT 3'
3' TTCAAATCTAGTCTTAAGCACTTTCGTCTTCAACAGACTATTAG. . .(82 nt). .GGTTCCsGAAATGAGCTACTCAGGGAATAGCACTA 5'
BsmFI

FIG. 5D

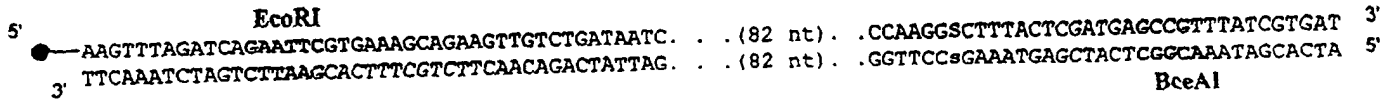

5'
EcoRI
AAGTTTAGATCAGAATTCGTGAAAGCAGAAGTTGTCTGATAATC. . .(82 nt). .CCAAGGSCTTTACTCGATGAGCCGTTTATCGTGAT 3'
3' TTCAAATCTAGTCTTAAGCACTTTCGTCTTCAACAGACTATTAG. . .(82 nt). .GGTTCCsGAAATGAGCTACTCGGCAAATAGCACTA 5'
BceAI

FIG. 6A

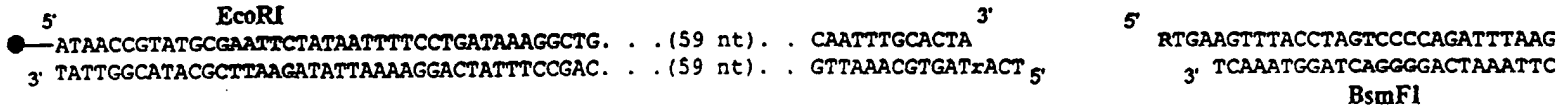

5'
EcoRI
3'
ATAACCGTATGCGAATTCTATAATTTTCCTGATAAAGGCTG. . .(59 nt). . CAATTTGCACTA
3' TATTGGCATACGCTTAAGATATTAAAAGGACTATTTCCGAC. . .(59 nt). . GTTAAACGTGATxACT 5'
5'
RTGAAGTTTACCTAGTCCCCAGATTTAAG
3' TCAAATGGATCAGGGGACTAAATTC
BsmFI

FIG. 6B

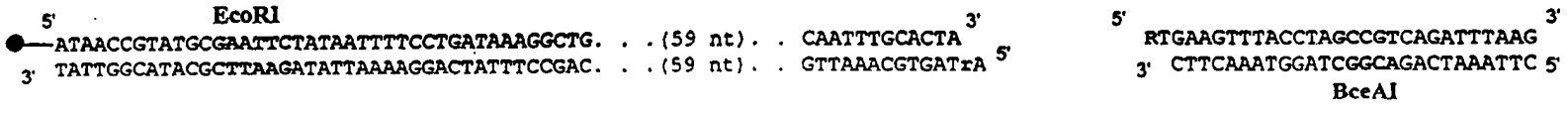

5'
EcoRI
3'
ATAACCGTATGCGAATTCTATAATTTTCCTGATAAAGGCTG. . .(59 nt). . CAATTTGCACTA
3' TATTGGCATACGCTTAAGATATTAAAAGGACTATTTCCGAC. . .(59 nt). . GTTAAACGTGATxA 5'
5'
3'
RTGAAGTTTACCTAGCCGTCAGATTTAAG
3' CTTCAAATGGATCGGCAGACTAAATTC 5'
BceAI

FIG. 6C

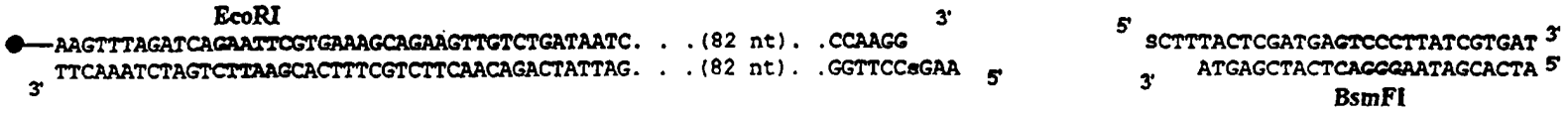

EcoRI
AAGTTTAGATCACGAATTCGTGAAAGCAGAAGTTGTCTGATAATC. . .(82 nt). .CCAAGG
TTCAAATCTAGTCTTAAGCACTTTCGTCTTCAACAGACTATTAG. . .(82 nt). .GGTTCCsGAA
BsmFI

FIG. 6D

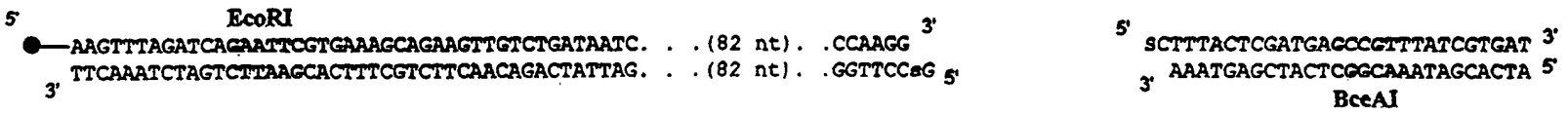

EcoRI
AAGTTTAGATCACGAATTCGTGAAAGCAGAAGTTGTCTGATAATC. . .(82 nt). .CCAAGG
TTCAAATCTAGTCTTAAGCACTTTCGTCTTCAACAGACTATTAG. . .(82 nt). .GGTTCCsG
BceAI

FIG. 7A

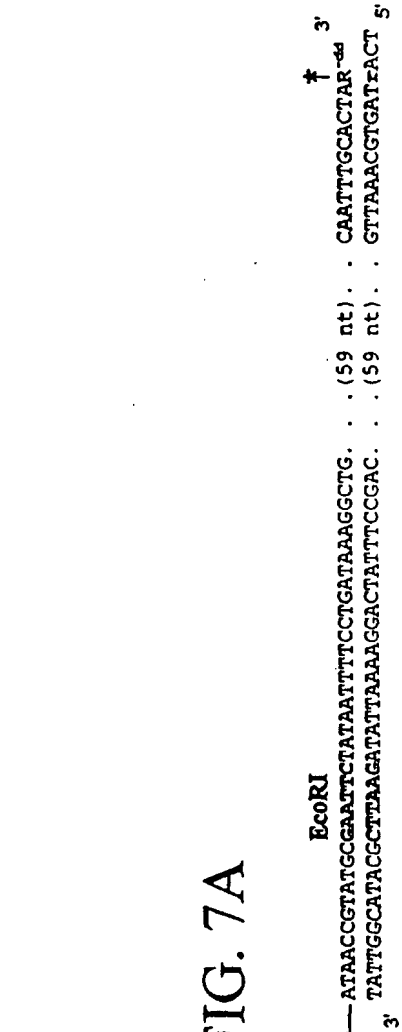

EcoRI
5'
ATAACCGTATGCGAATTCTATAATTTTCCTGATAAAGGCTG. . .(59 nt). . CAATTTGCACTAR-dd 3'
TATTGGCATACGCTTAAGATATTAAAAGGACTATTTCCGAC. . .(59 nt). . GTTAAACGTGATYACT 5'
3'

FIG. 7B

EcoRI
5'
ATAACCGTATGCGAATTCTATAATTTTCCTGATAAAGGCTG. . .(59 nt). . CAATTTGCACTAR-dd 3'
TATTGGCATACGCTTAAGATATTAAAAGGACTATTTCCGAC. . .(59 nt). . GTTAAACGTGATYA 5'
3'

FIG. 7C

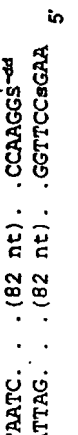

EcoRI
5'
AAGTTTAGATCAGAATTCGTGAAAGCAGAAGTTGTCTGATAATC. . .(82 nt). .CCAAGGS-dd 3'
3' TTCAAATCTAGTCTTAAGCACTTTCGTCTTCAACAGACTATTAG. . .(82 nt). .GGTTCCsGAA 5'

FIG. 7D

EcoRI
5'
AAGTTTAGATCAGAATTCGTGAAAGCAGAAGTTGTCTGATAATC. . .(82 nt). .CCAAGGS-dd 3'
3' TTCAAATCTAGTCTTAAGCACTTTCGTCTTCAACAGACTATTAG. . .(82 nt). .GGTTCCsG 5'

FIG. 7E

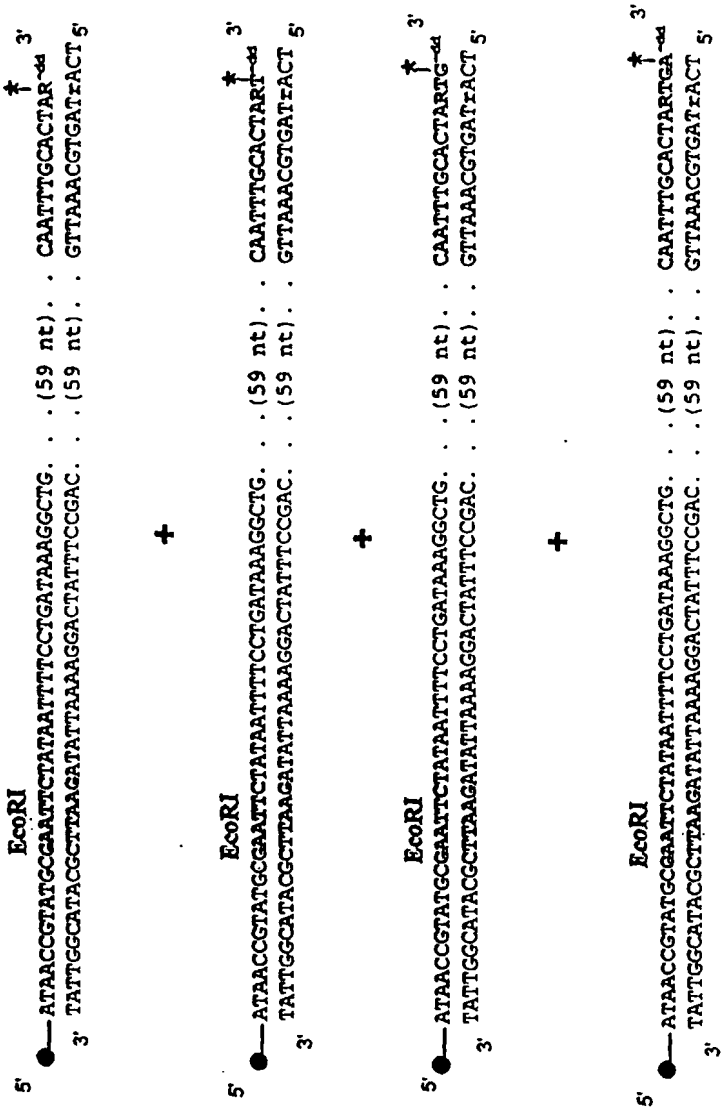

EcoRI
5'
ATAACCGTATGCGAATTCTATAATTTTCCTGATAAAGGCTG. . .(59 nt). . CAATTTGCACTAR
3' TATTGGCATACGCTTAAGATATTAAAAGGACTATTTCCGAC. . .(59 nt). . GTTAAACGTGATrACT 5'
+
EcoRI
5'
ATAACCGTATGCGAATTCTATAATTTTCCTGATAAAGGCTG. . .(59 nt). . CAATTTGCACTART
3' TATTGGCATACGCTTAAGATATTAAAAGGACTATTTCCGAC. . .(59 nt). . GTTAAACGTGATrACT 5'
+
EcoRI
5'
ATAACCGTATGCGAATTCTATAATTTTCCTGATAAAGGCTG. . .(59 nt). . CAATTTGCACTARTG
3' TATTGGCATACGCTTAAGATATTAAAAGGACTATTTCCGAC. . .(59 nt). . GTTAAACGTGATrACT 5'
+
EcoRI
5'
ATAACCGTATGCGAATTCTATAATTTTCCTGATAAAGGCTG. . .(59 nt). . CAATTTGCACTARTGA
3' TATTGGCATACGCTTAAGATATTAAAAGGACTATTTCCGAC. . .(59 nt). . GTTAAACGTGATrACT 5'

FIG. 8A

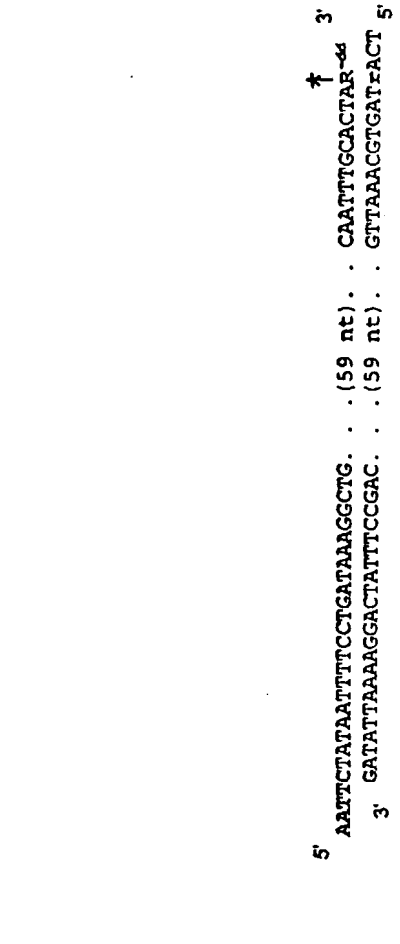

5'
ATAACGTATGCG
3'
3'
TATTGGCATACGCTTAA
5'
5'
AATTCTATAATTTTCCTGATAAAGGCTG. . .(59 nt). . CAATTTGCACTAR-dd
3'
3' GATATTAAAAGGACTATTTCCGAC. . .(59 nt). . GTTAAACGTGAT=ACT
5'

FIG. 8B

5'
ATAACGTATGCG
3'
3'
TATTGGCATACGCTTAA
5'
5'
AATTCTATAATTTTCCTGATAAAGGCTG. . .(59 nt). . CAATTTGCACTAR-dd
3'
3' GATATTAAAAGGACTATTTCCGAC. . .(59 nt). . GTTAAACGTGAT=A
5'

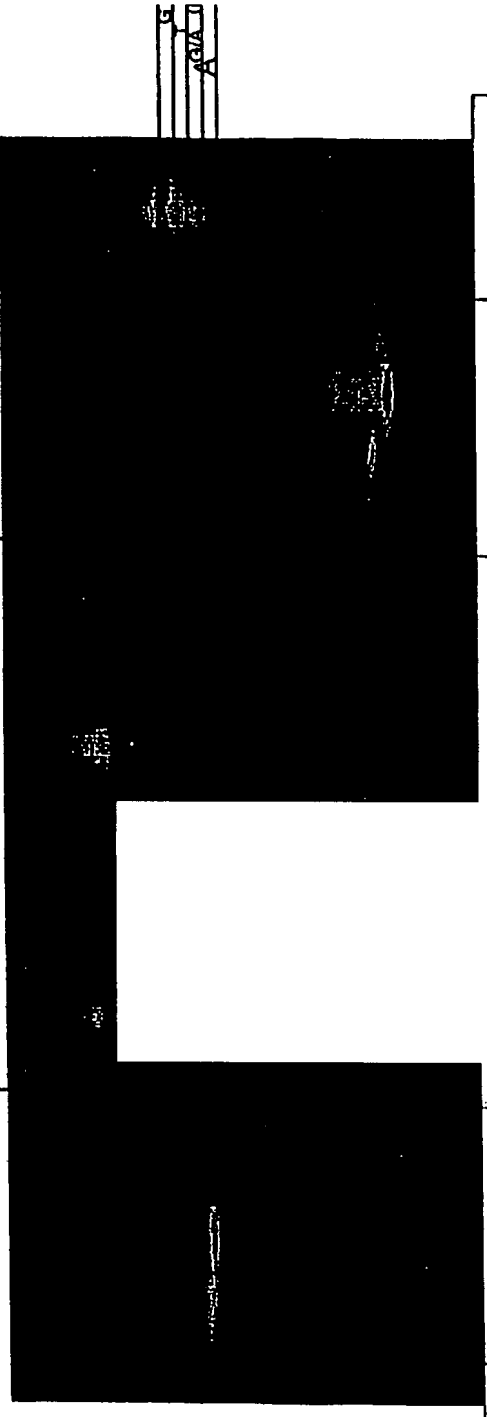
FIG. 8C

5'
AAGTTTAGATCAG 3'
3' TTCAAATCTAGTCTTAA 5'
5'
AATTCGTGAAAGCAGAAGTTGTCTGATAATC. . . (82 nt). .CCAAGGS
3' GCACTTTCGTCTTCAACAGACTATTAG. . . (82 nt). .GGTTCCsGAA 5'
3'

FIG. 8D

5'
AAGTTTAGATCAG 3'
3' TTCAAATCTAGTCTTAA 5'
5'
AATTCGTGAAAGCAGAAGTTGTCTGATAATC. . . (82 nt). .CCAAGGS
3' GCACTTTCGTCTTCAACAGACTATTAG. . . (82 nt). .GGTTCCsG 5'
3'

FIG. 9A FIG. 9B FIG. 9C FIG. 9D



Nucleotides	G A T C	G A T C	G A T C	G A T C
SNP	HC21S00027	TSC0095512	TSC0264580	HC21S00027
Restriction enzyme site on second primer	BceA I	BceA I	BsmF I	BsmF I
5' Overhang	3'A T r A 5'	3' G C s G 5'	3' G C s GAA 5'	3' T 10/14 cut A r CCC 5' 3'A 11/15 cut T A r CC 5'	3'A 10/14 cut T r ACT 5' 3'T 11/15 cut AT r AC 5'
R	G/A		A/C	G/A
S	G/A	G/C	G/C	

FIG. 10

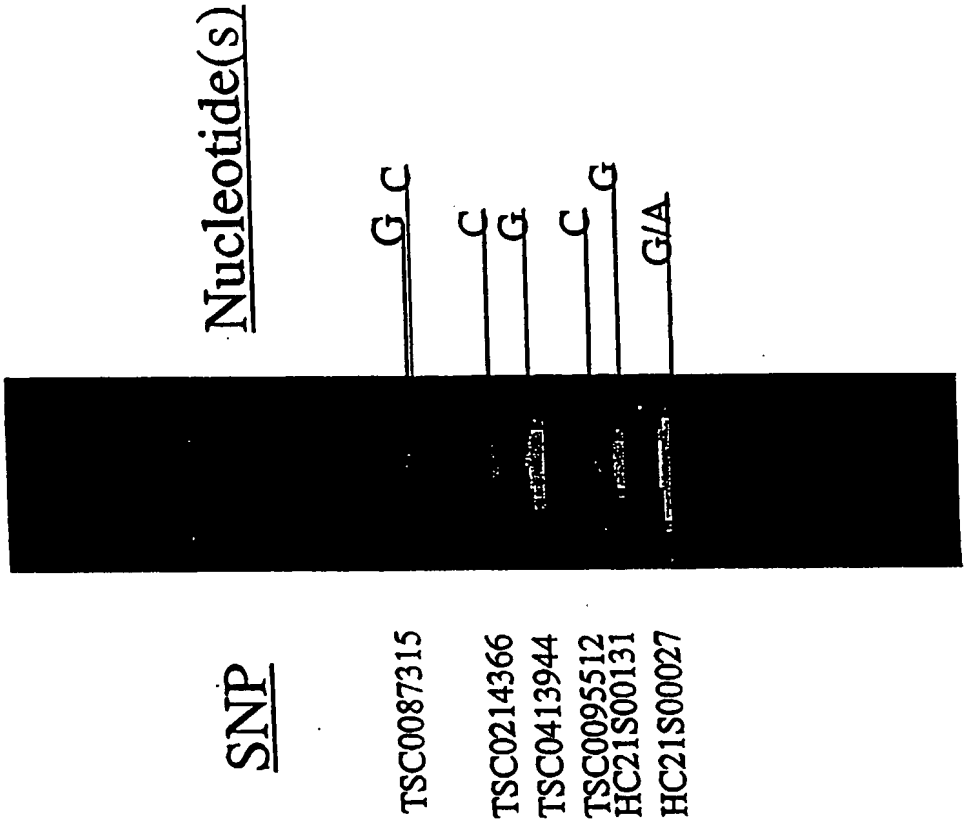

Nucleotide(s)
G C
C G
C G
G/A
SNP
TSC0087315
TSC0214366
TSC0413944
TSC0095512
HC21S00131
HC21S00027

FIG.11A

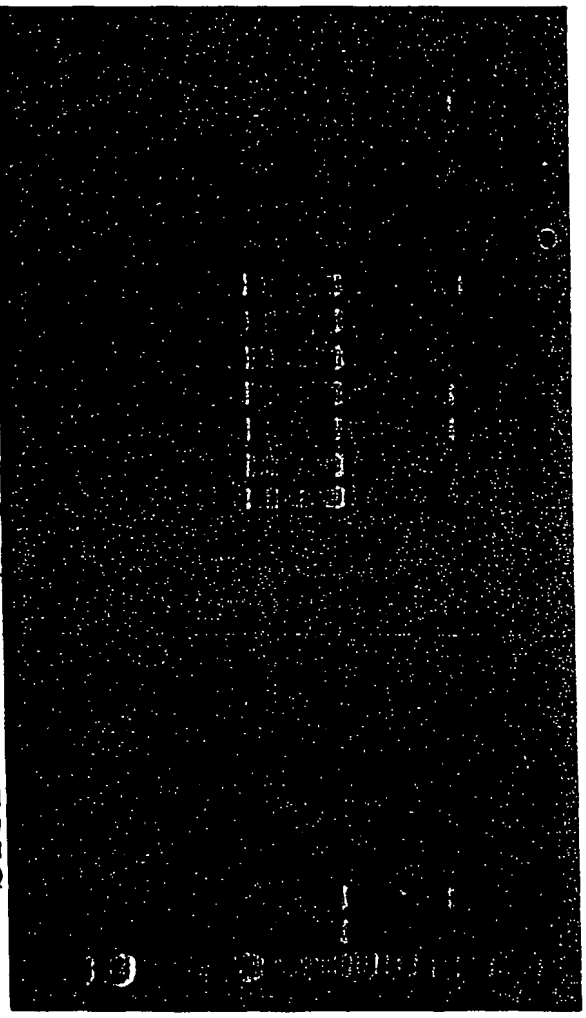

SRY Gene
Cystic Fibrosis Gene
Lane
M 1 2 3 4 5 6 7 8 9 10 12 13 14 15 16 17 18 19 20 21 22 23

FIG. 11B

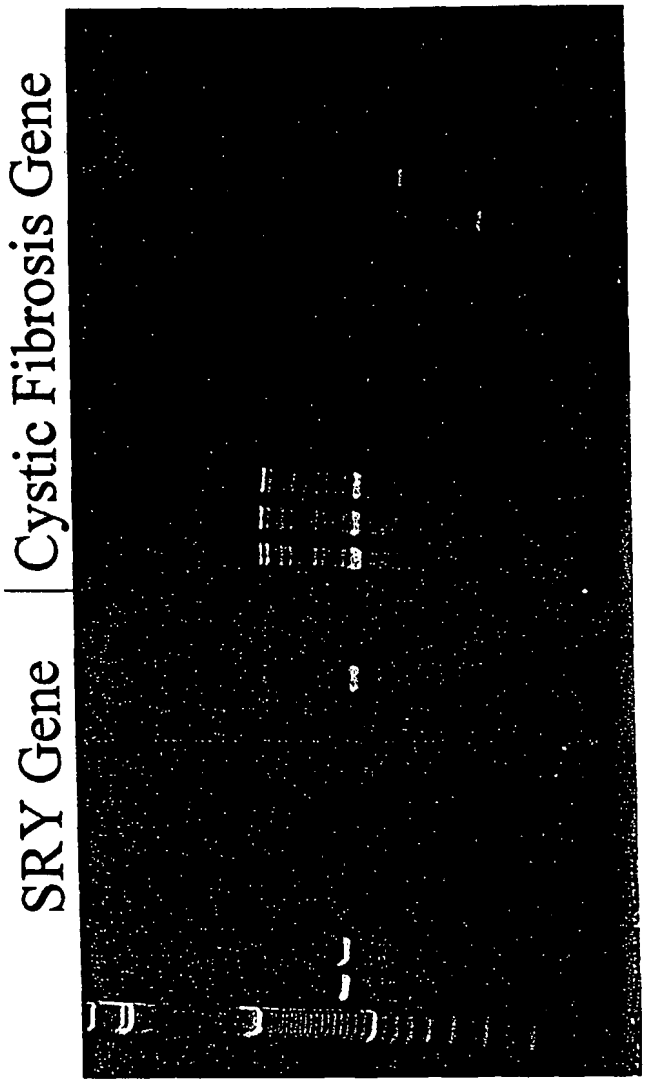
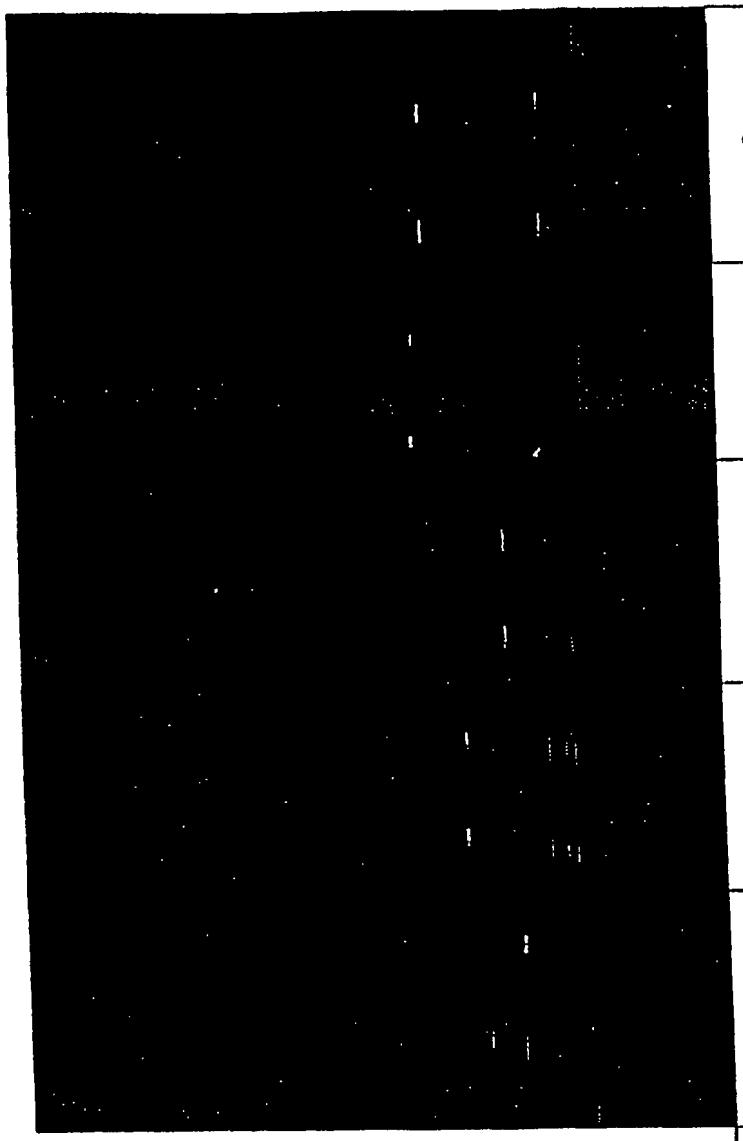

SRY Gene
Cystic Fibrosis Gene
Lane
M 1 2 3 4 5 6 7 8 9 10 12 13 14 15 16 17 18 19 20 21 22 23

FIGURE 12



Chromosome	21		21		13		13		13	
SNP	TSC 0115603		TSC0309610		ss813773		TSC0198557		TSC0200347	
Nucleotide	T	A	T	C	A	C	T	C	T	C
Nucleotide Ratio	36:63		66:33		46:54		49:51		50:49	

FIG. 13

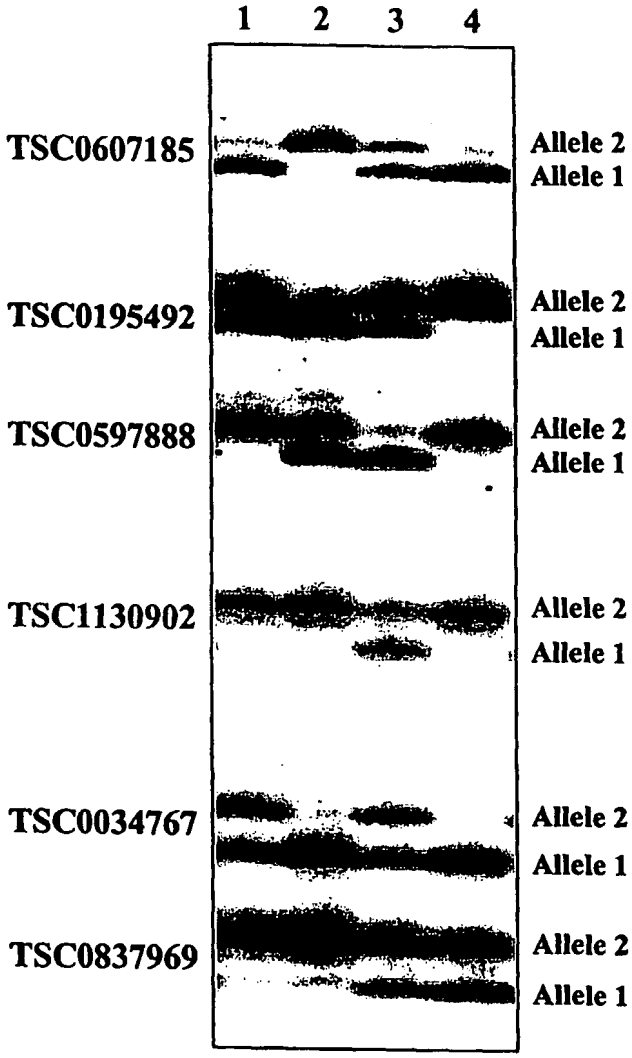
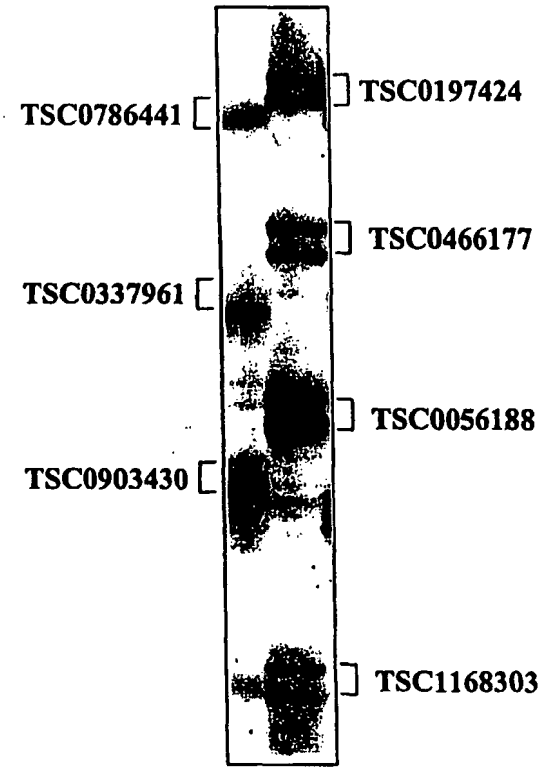

1
2
3
4
TSC0607185
Allele 2
Allele 1
TSC0195492
Allele 2
Allele 1
TSC0597888
Allele 2
Allele 1
TSC1130902
Allele 2
Allele 1
TSC0034767
Allele 2
Allele 1
TSC0837969
Allele 2
Allele 1

FIG. 14


TSC0786441
TSC0197424
TSC0466177
TSC0337961
TSC0056188
TSC0903430
TSC1168303

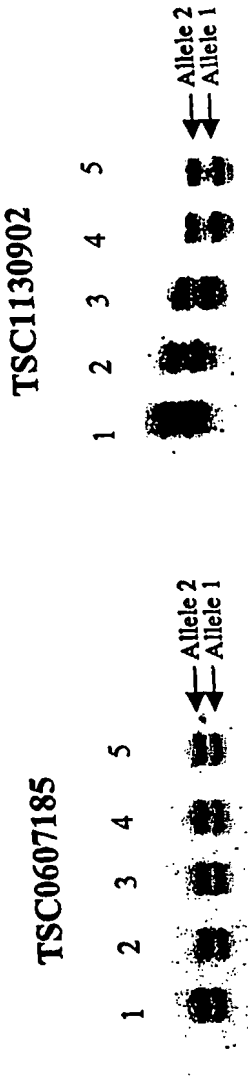
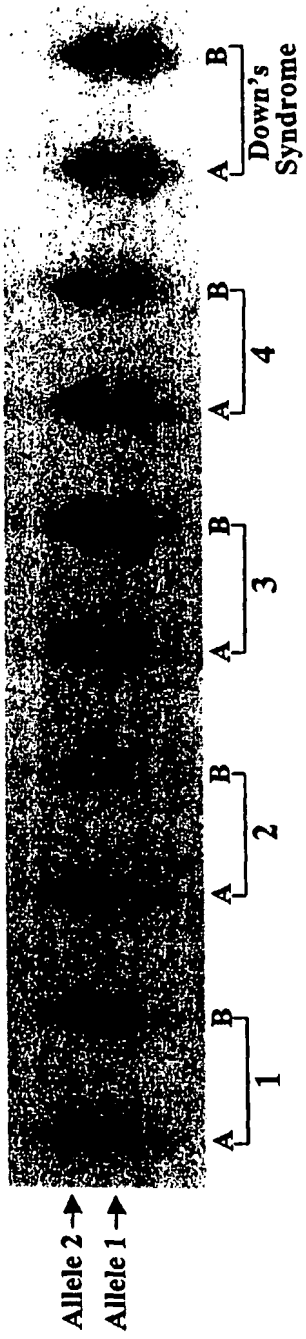

TSC1130902
1 2 3 4 5
Allele 2
Allele 1
TSC0607185
1 2 3 4 5
Allele 2
Allele 1

FIG.15

FIG. 16



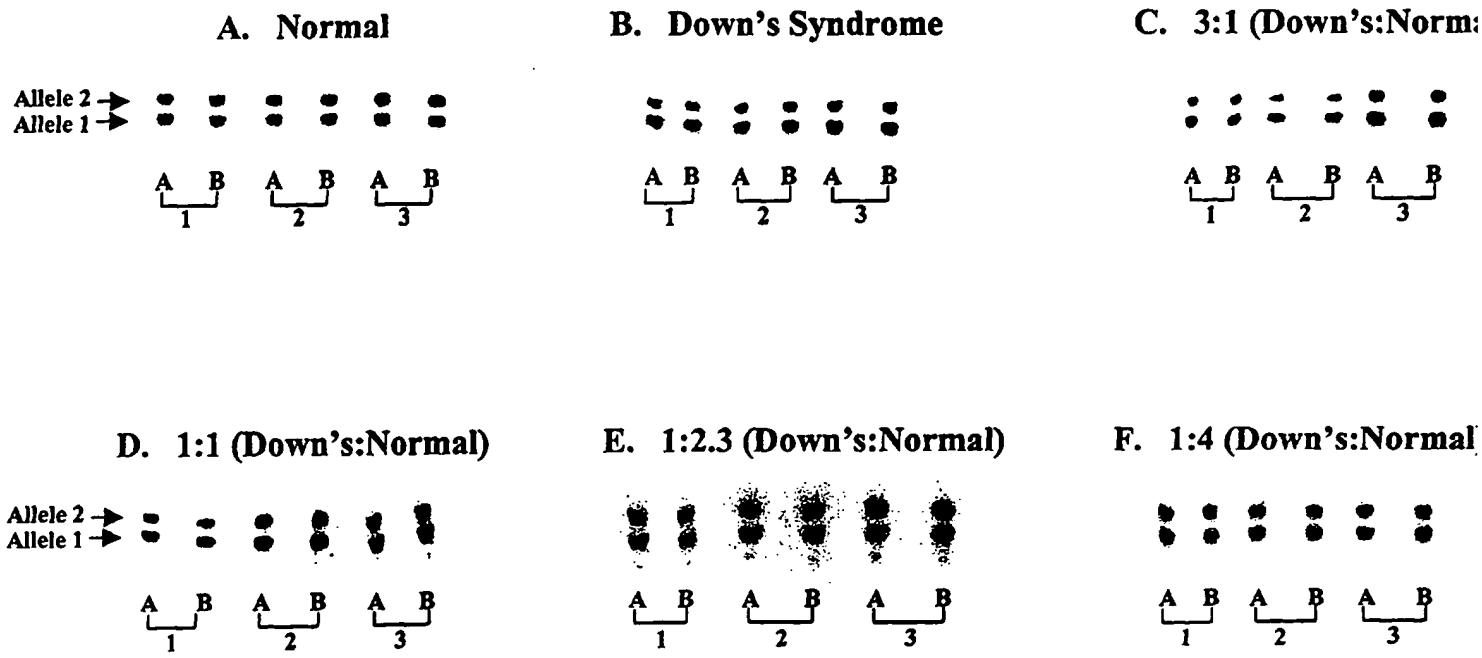

A. Normal
B. Down's Syndrome
C. 3:1 (Down's:Norm
D. 1:1 (Down's:Normal)
E. 1:2.3 (Down's:Normal)
F. 1:4 (Down's:Normal
Allele 2
Allele 1
A B A B A B
1 2 3

FIG. 17

FIG. 18

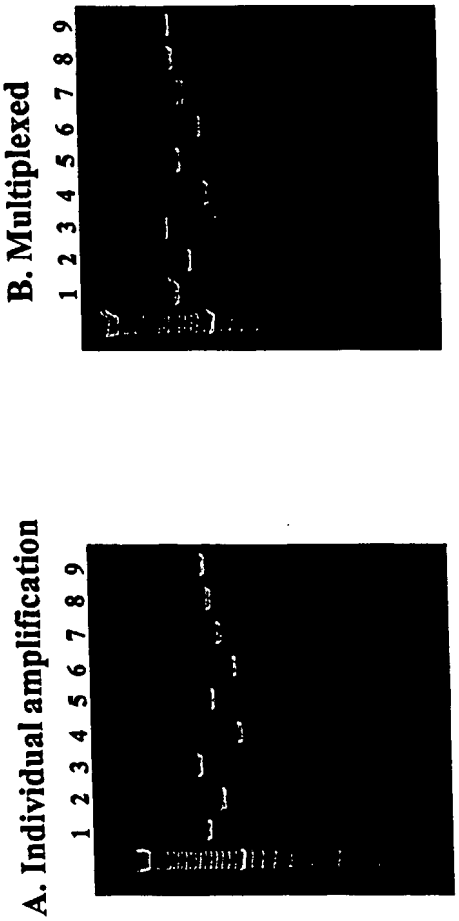

A. Individual amplification
1 2 3 4 5 6 7 8 9
B. Multiplexed
1 2 3 4 5 6 7 8 9

FIG. 19

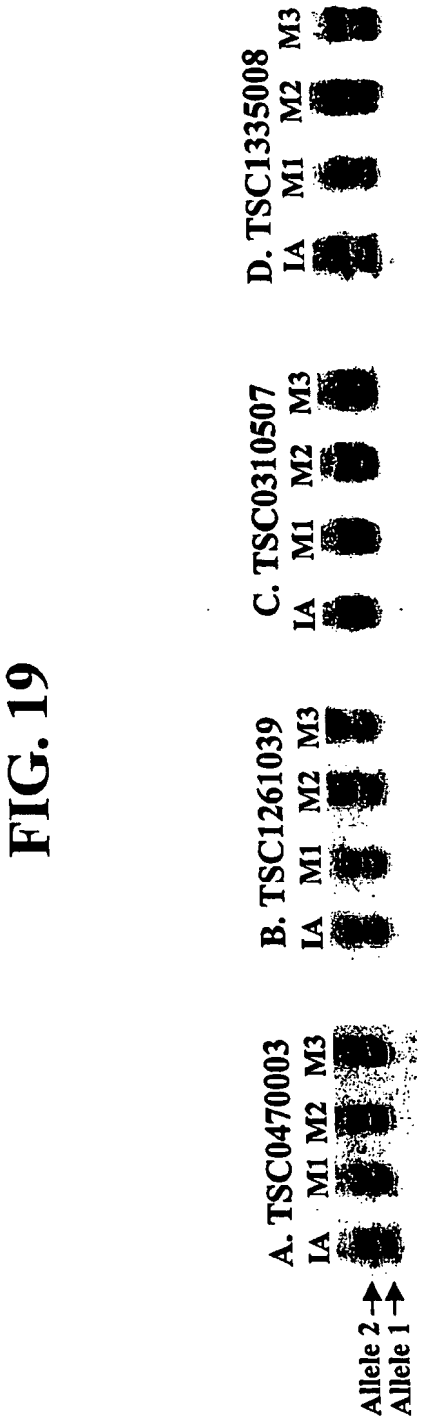
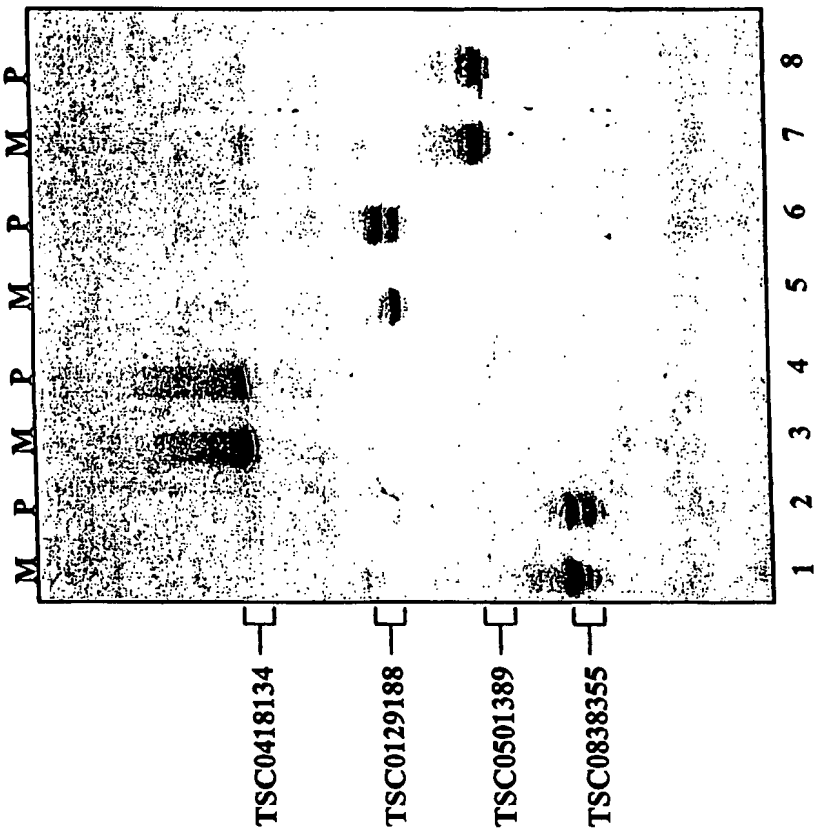

A. TSC0470003
IA M1 M2 M3
B. TSC1261039
IA M1 M2 M3
C. TSC0310507
IA M1 M2 M3
D. TSC1335008
IA M1 M2 M3
Allele 2
Allele 1

FIG. 20


M P M P M P M P
1 2 3 4 5 6 7 8
TSC0418134
TSC0129188
TSC0501389
TSC0838355

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/06198

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12Q 1/68; C07H 21/02, 21/04
US CL : 435/6; 536/23.1, 24.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6; 536/23.1, 24.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	US 2003/0054386 A1 (ANTONARAKIS et al.) 20 March 3003 (20.03.2003), see at least the abstract.	35 and 66
A,P	US 3003/0082576 A1 (JONES et al.) 01 May 2003 (01.05.2003) see at least the abstract.	1-66
A	UGOZZOLI et al. Detection of specific alleles by using allele-specific primer extension followed by capture on solid support. GATA. 1992, Vol. 9, No. 4, pages 107-12, see at least the abstract and Figure 1 on page 110.	1-66
A	KWOK, P.-Y. Methods for genotyping single nucleotide polymorphisms. Annual Reviews in Genomics and Human Genetics. 2001, Vol. 2, pages 235-258, see the entire	1-66
A	SHI, M.M. Enabling Large-scale pharmacogenetic studies by high-throughput mutation detection and genotyping technologies. Clinical Chemistry. 2001, Vol. 47, No. 2, pages 164-172, see the entire document.	1-66
A,P	US 6,475,736 A (STANTON, JR.) 05 November 2002 (05.11.2002) see the entire document.	1-66
A	US 5,831,065 A (BRENNER) 03 November 1998 (03.11.1998) see the entire document.	1-66

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 July 2003 (31.07.2003)	02 SEP 2003
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer Ethan Whisenant, Ph.D. Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/06198

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I, claim(s) 1-35, drawn to a method for detecting a chromosomal abnormality.

Group II, claim(s) 36-65 drawn to a method for determining the sequence of a locus of interest on fetal DNA.

Group III, claim(s) 66, drawn to a kit comprising a set of primers and a set of instructions.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or a corresponding special technical features.

Claims 1-35 are related in that they require quantitating the relative amounts of the alleles at a heterozygous locus of interest wherein the relative amount is expressed as a ratio and wherein said ratio indicates the presence or absence of a chromosomal abnormality. Claim(s) 36-66 lack this special technical feature which distinguishes Claims 1-35 over the prior art. Therefore, Claim(s) 36-66 lack unity with Claim(s)1-35.

The applicant is advised that there is no right to protest the lack of unity for any groups not paid for; AND any protest must be filed no later than 15 days from the mailing of the Search Report (PCT/ISA/210).

Continuation of B. FIELDS SEARCHED Item 3:
USPATFULL and EUROPATFULL via EAST; CAplus and Medline via STN

search terms: Genotyp$ and SNPs or Single Nucleotide Polymorphism? allele?, chromosom$ abnormality and/or inversion and/or translocation and/or deletion? and/or break? and ratio?; TypeIIs and/or restriction enzyme and/or endonuclease, fill in or fill-in and sequenc$; fetal DNA, cell lysis inhibitor and/or glutaraldehyde, formaldehyde, formalin.